STUDIES ON TUMOR-ASSOCIATED IMMUNOGLOBULINS

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To my wife

KATHLEEN

who helped me finish this study

And to my family

BESSILIOS, MARY, ROSINE and my Parents,

who helped me begin it.

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ABSTRACT

A direct and indirect radio-immune antiglobulin tests (RIAT) were developed and used to study tumor-associated immunoglobulin (TAIg) levels in transplanted freshly excised solid tumors. The studies were performed on 8-12 week old inbred CBA mice. The tumors investigated were grown from freshly excised and cultured CCH1 and T3 fibrosarcoma tumor cells.

The RIATs developed were based on isotopic antiglobulin tests which had been used in this department to determine circulating anti-tumor antibodies in tumor-bearing mice. They were modified and improved in order to be used to detect *in vivo* bound TAIgs in solid tumors. The indirect RIAT developed was used in both quantitative and qualitative TAIg studies. The more sensitive direct RIAT was used solely in quantitative studies on TAIgs.

In order to optimize the test a number of variables were investigated. These included the method of tumor cell preparation and the influence of reagent concentration, tumor cell number, and the period of incubation. Furthermore, attempts were made to reduce nonspecific binding of reagents by precoating the plastic plates and tubes with various protein containing media. As a result of these investigations, techniques were developed which were sufficiently sensitive for semi-quantitative and semi-qualitative studies.

In vivo bound TAIgs in solid tumors and its variation with age and size of tumors was assessed by both the direct and indirect RIATs. The more immunogenic CCH1 tumors were found to possess higher levels of TAIgs than the less immunogenic T3 tumors. Moreover, tumors produced by the injection of tumor cells contaminated with host cells were found to possess higher levels of TAIgs than tumors produced by cultured tumor cells which are devoid of host cells. Similarly, tumor cells prepared in different ways(enzyme vs mechanical) were found to produce tumors of different TAIg content. In addition, the *in vivo* binding of TAIgs was found in general to vary positively with the age and size of the tumors. The injection of 1-5 million tumor cells was found to produce larger tumors of higher TAIg content than the injection of 0.1-0.5 million tumor cells. Similar effects were noted whether the tumors were compared in different mice or on opposite limbs of the same mouse. Comparative results were also obtained by a double radio-immunoassay test which was carried out in parallel with the RIATs. The amount of *in vivo* bound TAIg in solid tumors was thus found to be dependent on immunogenicity, method of maintenance (ie. *in vivo* or *in vitro*), and the method of preparation of the tumor cell inoculum.

The cellular basis of TAIgs was studied in immuno-stimulated and immuno-deficient mice. Intraperitoneal *C.parvum* (CP) administration resulted in an increase of TAIgs in both CCH1 and T3 tumor models, though this effect was more marked in the former. Moreover, additional studies revealed that CP treated CCH1 tumors had higher levels of cell-surface bound IgA, IgG2b, and IgG3 than non-treated CCH1 tumors. Parallel qualitative studies on CCH1 tumor extracts showed that CP treated tumors also had more IgG1, IgA and IgM.

Studies were also performed in thymectomized and nude mice in order to assess the effect of T-cell deprivation on the in vivo binding of TAIgs. The TAIg content in tumors grown in T-cell deficient mice was shown not to differ from tumors grown in normal mice. Preliminary experiments were also conducted on mice treated with gold salts in order to determine the influence of macrophages in TAIg binding. No significant difference was obtained between tumors grown in gold-salt treated mice and nontreated mice. Moreover, whole body irradiation (400 rads) 24 hrs. before the injection of tumor cells was not found to appreciably effect the in vivo binding of TAIg.

The significance of these results and their relevance to tumor immunodiagnosis and immunotherapy are discussed.

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" Science does not need to apologize for its inability to offer us certainties; that should rather be its boast, as signalizing its prospects for further advance. Yet I must at least confess to the certitude that it has advanced and is advancing, which can only mean that some true knowledge of reality pervades its results, and that the sum of such knowledge increases as time goes on. "

John Baillie

The Sense of the Presence of God, 1962.

PREFACE

In the early part of this century, the eventual immunological control of cancer seemed possible since experiments with outbred animals showed that tumor cells could be rejected when transferred into various animals. However, studies on inbred animals in the 1930's indicated that these early hopes were premature. For the rejection of tumor cells was found to be due to normal transplantation antigens present on these cells. It was only after two decades when the existence of tumor-specific transplantation antigens was demonstrated that immunology gradually became an integral part of cancer research.

INTRODUCTION

1. PROGRESS IN TUMOR IMMUNOLOGY

Extensive research in tumor immunology during the 1970's must have brought little comfort to those immunologists who began the decade with great hopes for the immunological control of cancer. The accumulated experimental and clinical data in tumor immunology unravelled a field of immense complexity, to the extent that more questions were raised than answered. Immunologists began the last decade by asking the question, "does the immune system prevent tumor growth?" (Burnet, 1970), and ended the decade with the question, "does the immune system enhance tumor growth?"; a question which was inspired by the immuno-stimulation theory (Prehn, 1977). In addition, certain anti-tumor antibodies, immune-complexes, and suppressor T cells were demonstrated to inhibit the cytotoxic action of host immunocompetent cells against tumors.

However, in so far as the obtained data indicated the complexity of tumor immunology, it also revealed a significant relationship between the immune system and tumor growth. Tumor associated antigens were shown to exist in many chemically and virally induced tumors; macrophages, T cells, and certain tumor specific antibodies were found to inhibit tumor growth; and various immunostimulants were shown to improve the prognosis of cancer patients.

One can envisage that during the 1980's research in tumor immunology will concentrate on the elucidation of the immunological mechanisms that promote or inhibit tumor growth. Although a great deal

of time and work might precede the understanding of these mechanisms, the ultimate contribution of tumor immunology in the prevention and control of cancer remains in little doubt.

1.1 Tumor-associated antigens

The "raison d'être" of tumor immunology is the existence of tumor-associated antigens. Without the latter, research in tumor immunology could hardly be expected to achieve its practical aspirations, which include immunotherapy and immunodiagnosis. Since tumor-associated antigens were shown to exist on chemically and virally induced tumors, as well as on some spontaneous tumors (Shimkin, 1977), the significance of tumor immunity in these tumors has been in little doubt. On the other hand, the unequivocal demonstration of tumor-associated antigens where it is of primary concern, i.e. in human and spontaneous animal tumors, has been unsuccessful so far (Weiss, 1977). One of the reasons behind the latter is believed to be due to the presence of the antigens on such tumors in cryptic form. This possibility has been reinforced by recent results which have shown the conversion of non-antigenic tumor cells to antigenic tumor cells by enzymatic treatment (Bekesi and Holland, 1976). However, not all cell-surface manipulations have been found to produce this effect. In one study, protein cross-linking on the surface of tumor cells was found to inhibit the immunogenecity of the tumor cells (Price et.al., 1979). It is interesting that although the latter cells lost their immunogenecity, their antigenicity was maintained. This revealed the important role the structure of cell-surface antigens played in eliciting the immune response.

Another reason behind the absence of antigens on spontaneous tumors could be the rapid shedding of antigens from the surface of tumor cells. A third reason could be that antigens are too weak to be detected by conventional techniques. These hypotheses give credence to the attempts that are presently being made to expose, isolate, and purify tumor-associated antigens on spontaneous experimental and human tumors.

Antigens on experimental tumors consist of three types, tumor specific antigens (TSA), tumor associated antigens (TAA), and tumor associated transplantation antigens (TATA) (Moore, 1978). At present, the existence of TSA cannot be stated with certainty. However, TAA have been demonstrated and they include foetal antigens, like carcinoembryonic and alpha-foeto protein, and cell surface antigens, induced by onco-RNA viruses. TATA, which occur on the tumor cell-surface, are capable of eliciting in vivo transplantation rejection to autochthonous tumor cells.

1.1.1 Chemically induced tumors : Methylcholanthrene, dimethylbenz (a) anthracene, ethyl carbamate and benzpyrene are examples of the chemicals that can induce tumors in animals. Whereas their mechanism of tumor induction and period of latency may vary, they all share the capacity to induce tumors which possess distinct TATA. Even different tumors induced in the same animal by the same chemical exhibit antigenic differences. However, antigenic cross-reactivity may be demonstrated and this could be attributed to "passenger virus" or "antigenic conversion" (Moore, 1978). In addition, serological studies have shown chemically induced tumors to possess surface TAA as well.

1.1.2 Virally induced tumors : Certain DNA and RNA viruses are known to induce tumors in animals. Examples of the former are simian papova-virus (SV40) and herpes simplex; and examples of the latter are murine leukemia virus (MuLV) and mouse mammary tumor virus. Unlike chemically induced tumors, common antigens do exist on different tumors induced by the same virus. Tumor antigens associated with DNA viruses include nuclear and cytoplasmic antigens, and cell-surface antigens; while tumor antigens associated with RNA viruses include diverse virion and virus induced antigens (Witz et.al., 1976 and Moore, 1978).

1.2 Tumor immunosurveillance

The discovery of transplantation antigens and their ability to elicit cellular immunity, and the demonstration of tumor-associated antigens in experimental tumors led to the emergence of the immunosurveillance theory. Introduced by Burnet in 1970, it stated that tumors were continuously arising in the human body, but that the majority of them were eliminated at the initial stages by the immune system. In other words, the immunosurveillance theory predicted an increase of tumor incidence in immunosuppressed individuals, and the improvement of prognosis by immunostimulants. After a decade of intensive research in tumor immunology, the immunosurveillance theory still has its advocates and opponents.

The opponents of the immunosurveillance theory base their argument on three main points: monoclonality of tumors, absence of major differences in antigenic strength between in vivo and in vitro induced tumors, and lack of significant increases of tumor incidence

in immunosuppressed animals. Moller and Moller (1978) argue that the monoclonality of tumors in neoplasia casts doubt upon the immuno-surveillance theory, for if the latter was true then the failure of the immune system should give rise to polyclonal tumors. Moreover, according to the immunosurveillance theory, only weakly antigenic tumor-cells would escape the immune system suggesting, therefore, that tumors induced spontaneously *in vitro* would be antigenically stronger. However, no significant differences in antigenic strength between *in vivo* and *in vitro* induced tumors have been observed (Evans *et. al.*, 1975 and Moller and Moller, 1976).

Apart from DNA viral tumors, no significant increase of tumor incidence has been noted in immunosuppressed animals (Stutman, 1975). Although a similar assessment of tumor incidence in Man remains to be made, the latter observation gives weight to the arguments put forward by the opponents of the immunosurveillance theory. Moreover, a further set-back to the immunosurveillance theory has come from the emergence of the immunostimulation theory which predicts that, far from inhibiting tumors, the immune system might actually contribute to the growth of tumors (Prehn, 1976).

Advocates of the immunosurveillance theory base their arguments on the following : the immunosuppressive properties of oncogenic viruses; the apparent increase of tumor incidence in immunosuppressed patients; improved prognosis of cancer patients undergoing immunotherapy; and non-T cell surveillance against tumors in nude mice. Friedman and Ceglowski (1973) postulated that immunosuppression by viruses was responsible for many of the tumors they produce. The role of immuno-suppressive factors in the etiology of cancer was expounded further by Kamo and Friedman (1977) who stressed the importance of the immune

system in the host-parasite relationship that exists during the development and progression of the malignant process. The apparent increase of tumor incidence in immunosuppressed patients, especially those who have undergone organ transplantation (Videback, 1973 and Penn and Starzl, 1973), has helped to reinforce the argument of the advocates of the immunosurveillance theory.

The significance of the immune system in cancer was further revealed by various immunotherapeutic trials. The administration of immunostimulants such as Corynebacterium parvum and Bacillus-Calmette Guerin together with chemotherapy, irradiation and/or surgery has been found in some cases to improve the prognosis of cancer patients (Mastrangelo et. al., 1976). Finally, nude mice, whose lack of increase in tumor incidence has strongly supported the opponents of the immuno-surveillance theory, have been shown to possess strong non-T cell mediated surveillance mechanisms (Kiessling et. al., 1975; Herberman et.al., 1975; Kiessling et. al., 1976 and Klein and Klein 1977). The possible implication of non-immunological factors in the defence of newborn mice against tumors was also shown by Zinzar et. al., (1976 and 1978).

In conclusion, the immunosurveillance theory appears to be lingering on in spite of the doubt cast upon it by substantial contra-indicative experimental data. Ultimately, the relevance or irrelevance of the immunosurveillance theory might be revealed, but only after sufficient research has been conducted into human cancer. Meanwhile, the importance of the immune system in the progression of tumor growth continues to be supported by experimental evidence.

1.3 The immune system and tumor growth

When one considers the various types of tumors, the different species of experimental animals, and the diverse methods of immuno-assays that have been used, the delay in the achievement of conclusive results in tumor immunology becomes understandable. However, this delay does not imply that the efforts of tumor immunologists have been in vain. On the contrary, their intensive research has shown the importance of both specific and non-specific humoral and cellular immunity in tumor growth. Anti-tumor antibodies were shown to exist in the sera of tumor-bearing animals and cancer patients (Jose and Skvaril, 1974; Ran et. al., 1976; Lewis et. al., 1976; Heimen and Klein 1976; Haskill et. al., 1977; and Gupta et. al., 1979) and within their tumor (Vanky et. al., 1975; Dorval et. al. 1976; Witz 1977; Gersth et. al., 1977; Witz and Moav, 1978; Moav et. al., 1978; Ehrlich and Witz, 1979 McCoy et. al., 1979 and James et. al., 1979).

Similarly, specific cellular immunity against tumors was shown to exist in the peripheral blood of tumor bearing animals (Bruce et. al., 1976; Plata et. al., 1976 and Holden et. al., 1976) and cancer patients (Hellstrom et. al., 1971 and Jondal et. al., 1975), and within the tumors of experimental animals (Holden et. al., 1976 and Russell et. al., 1976a & b). It must be stated, however, that the association of specific cellular immunity in cancer patients has been less apparant than that observed in experimental animals (Takasugi et. al. 1973 and Bean et. al. 1975). Macrophages too were shown to play an important role in tumor growth inhibition (James et. al., 1977; Miller and Feldman, 1977; Haskill, 1977; Lohmann-Mathes et. al., 1979 and Domzig and Lohmann-Mathes, 1979).

Although the diverse data produced by the different experimental systems has hindered progress towards the elucidation of the mechanisms involved in tumor immunology, the results have shown the relationship between the immune system and tumor growth to be two sided. On the one hand, tumor growth was shown to be enhanced by the immune system and conversely, it was shown to be inhibited by the immune system. The types of immune cells or immunoglobulin classes associated with either effect have not been clearly determined so far. However, results in general have indicated that immunoglobulins and antigen-antibody complexes tend to enhance tumor growth, while T cells and macrophages tend to inhibit tumor growth (Klein, 1972; James et. al., 1975; James et. al., 1976; Fiedman, 1976; Baldwin and Price, 1976; Price and Baldwin, 1977; Johnson et. al., 1977; Witz, 1977;Shearer and Fink, 1977; Hofken et.al., 1978a; Hofken et.al., 1978b; Hofken et. al., 1978c; Shin et.al., 1978; and Price et. al., 1979).

It can be stated that, while the association of the immune system with tumor growth has been shown, only a glimpse of the mechanisms involved in tumor immunology has been obtained. Continued research in tumor immunology will, it is believed, elucidate these mechanisms and enable them to be utilized in the prevention and cure of cancer. The possibility of such an achievement is especially boosted by the encouraging results attained in the practical applications of tumor immunology.

1.4 The practical application of tumor immunology

Although tumor immunology has barely progressed beyond its preliminary stage, attempts have already been made to apply it in tumor immunodiagnosis and immunotherapy. In immunodiagnosis, anti-tumor antibodies have been utilized to forecast malignant growth (Ting, 1976), and to locate occult neoplasia (Terman et.al., 1975).

In immunotherapy, various immunostimulants have been used to improve the prognosis of cancer patients (James et. al., 1975; Mastrangelo et. al., 1976; Israel, 1976; Torisu et. al. 1976 and Gutterman et. al., 1976). Although not all trials have succeeded in extending the period of remission or preventing the regrowth of tumors, some trials have achieved very encouraging results. Moreover, anti-tumor antibodies have been used to enhance the specificity of anti-tumor drugs (Ghose and Blair, 1978).

The relevance of immunology to tumor growth is further expounded in the following sections. It will become apparent that the available data have paved the way for significant advances to be made in tumor immunology.

2. ANTI-TUMOR ANTIBODIES

The study of anti-tumor antibodies was first restricted to humoral antibodies in the sera of tumor bearing animals and cancer patients. Although circulating anti-tumor antibodies were demonstrated in the sera of tumor bearers, hardly any

concrete correlation was found between tumor growth *in vivo* and circulating anti-tumor antibodies. Consequently, research has been extended to anti-tumor antibodies found within the tumor, where TAIgs were found to exist on tumor cells and host cells. Some of these anti-tumor antibodies have been isolated and their behaviour studied both *in vitro* and *in vivo*. Although greater insight has been gained into the association of anti-tumor antibodies with tumor growth, and the practical benefits that can be accrued from them in tumor diagnosis and drug-guidance in chemotherapy, the precise role of anti-tumor antibodies has not yet been fully elaborated.

2.1 Circulating anti-tumor antibodies

Initial research on circulating anti-tumor antibodies produced contradictory results. On the one hand, they were found to block the anti-tumor action of host cells and enhance tumor growth (Bansal *et. al.*, 1972), while on the other hand, they were found to cooperate with host cells and inhibit tumor growth (Johnson *et. al.* 1977). What made the anti-tumor antibodies behave in one way or the other appeared to depend on the type of tumor, extent of tumor growth and the immunological state of the host. Moreover, it has become clear that anti-tumor antibodies are seldom in themselves responsible for the "blocking effect" (Price and Robins, 1978). For instance, although anti-tumor antibodies persist in the sera of mice after the excision of their tumors, post-surgical sera rarely exhibit a blocking effect.

Also, immune sera which bind to effector cells *in vitro* and block their subsequent action on tumor cells, are nonetheless capable of rejecting tumor growth *in vivo*. Therefore, factors other than anti-tumor antibodies appear to be responsible for the blocking effect, such as soluble tumor antigens, antigen antibody complexes (Coggin *et. al.*, 1974) and other tumor cell products (Dauphine *et. al.*, 1974; Keisari and Witz, 1975 and Lewis *et. al.*, 1976)

2.1.1 Circulating anti-tumor antibodies in experimental animals.

Although the role of circulating anti-tumor antibodies in tumor rejection or enhancement remains unclear, their existence in the sera of tumor-bearing animals has been well documented. In one study by Ran *et. al.* (1976), cytotoxic anti-tumor antibodies in mice appeared on the second week after the injection of tumor cells and remained high thereafter. In a similar study by Ting (1976), kinetics of the antibody response and its specificity was found to vary between different tumors. Mice with progressive tumors were found to have higher levels of circulating anti-tumor antibodies than mice with regressing tumors or no tumors at all. In the sera of mice bearing a methyl-cholanthrene induced sarcoma, significant levels of circulating anti-tumor antibodies appeared after excision (Harder and McKhann 1968).

Noncytolytic circulating anti-tumor antibodies were among the antibodies found in the sera of DBA/2j mice hyperimmunized against L-513-tumor cells (Goldstein *et. al.*, 1973). Haskill *et. al.* (1977) were able to demonstrate the presence of IgG1, IgG2a, IgG2b, IgA and IgM antibodies in the sera of DBA/2 mice bearing T1699 tumors. IgE has also been associated with tumors (Rosenbaum and Dwyer, 1977).

Robins (1975) showed the presence of circulating anti-tumor antibodies in the sera of mice bearing both ascitic and solid tumors. However, once the tumors in the latter became palpable, the antibodies disappeared from the corresponding sera. Similar results were noted in a review by Coggin et. al. (1974).

2.1.2 Circulating anti-tumor antibodies in cancer patients

Circulating anti-tumor antibodies have also been demonstrated in the sera of cancer patients, although their anti-tumor specificity remains less clear than the circulating anti-tumour antibodies in experimental animals. In most studies, the association of circulating antibodies with the tumor was observed by the ability of these antibodies to block the anti-tumor action of host cells. Steel et.al (1975) were able to decrease the blocking effect in the sera of patients after absorbing these sera with S.aureus protein A which is known to bind to certain IgG subclasses. Similarly, IgG1 and IgG3 were found to be associated with the blocking effect in the sera of children with neuroblastoma (Jose and Skvaril, 1974). Heimen and Klein (1976) were able to demonstrate immune complexes in the sera of patients with Burkitt's lymphoma and nasopharyngeal carcinoma, where IgG was found to be a principal component. Lewis et.al. (1976) asserted that anti-antibodies were an integral part of tumor associated antibodies. These anti-antibodies were thought to have both beneficial effects, when directed against blocking circulating anti-tumor antibodies and detrimental effects, when directed against cytolytic circulating anti-tumor antibodies.

2.1.3 *In vivo* and *in vitro* effects of circulating anti-tumor antibodies

As mentioned earlier, the *in vivo* and *in vitro* effects of circulating anti-tumor antibodies have been shown to correlate with tumor growth enhancement, on the one hand, and tumor growth inhibition, on the other. Bansal *et.al.* (1972) showed that the injection of sera obtained from rats carrying a progressively growing polyoma tumor into polyoma isografted rats enhanced the growth of the tumor. Tamerius *et.al.* (1975) found that the blocking factor in the sera of multiparous mice was associated with IgG2a and IgG2b. In contrast, Harris *et.al.* (1978) showed that isolated anti-tumor IgG1 antibody enhanced tumor growth *in vivo*, while whole antisera rarely did so. In another study, it was demonstrated that anti-SV 40 antisera which were positive in the *in vitro* antibody-dependent-cell-cytotoxicity, were not able to protect against SV40 tumor cell challenge (Prather *et.al.* 1979). In some instances, antibodies to normal cells have been found to enhance tumor growth. Heidrick *et.al.* (1978) found that a certain dilution of antibodies to normal skin cells which were cytotoxic to malignant cells *in vitro*, became tumor growth enhancers at a greater dilution.

There is ample evidence too for tumor growth suppressive properties of circulating anti-tumor antibodies. Johnson *et.al.* (1977a & b) demonstrated that allogeneic IgG1 anti-tumor antibody cooperated with macrophages and blood platelets to supress tumor growth *in vivo*. Miller and Feldman (1977), working with another tumor system, made similar observations; however, the suppressor antibody that cooperated with macrophages was found to be IgG2b. IgG2a, with or without macrophages, enhanced tumor growth. Farram *et.al.* (1978) showed that antitumor antibodies inhibited the growth of Meth.A tumors both *in vitro* and *in vivo*, probably through a complement dependent lysis of the tumor cells.

Cantrell et.al. (1976) further demonstrated that complement dependent cytotoxic anti-tumor antibodies which were produced after chemotherapy and immunotherapy, correlated well with improved prognosis.

In conclusion, it can be said that while research on circulating anti-tumor antibodies has been successful in associating these antibodies with the tumor, it has failed so far to elucidate the in vivo mechanisms involved, be it in tumor growth enhancement or inhibition.

2.2 In situ tumor-associated immunoglobulins

The failure to demonstrate a clear relationship between the role of circulating anti-tumor antibodies and tumor growth in animal models or cancer patients, prompted researchers to study in situ tumor-associated immunoglobulins (TAIgs). It was realized that the essential problem was not whether anti-tumor antibodies existed in the sera of mice and cancer patients, but whether such antibodies reached the tumor site at all and what happened thereafter in the tumor micro-environment.

One of the difficult tasks that confronted pioneer workers in this field was the development of sensitive techniques to detect immunoglobulins on the surface of freshly excised tumor cells. The routine preparation of tumor cells by enzyme digestion could not be used as the latter was known to remove surface bound TAIgs. The indirect antiglobulin test was one technique which had been used to study tumor cell surface antigens. Sparks et.al. (1969) developed the isotopic antiglobulin test introduced by Harder and McKhann (1968) to study humoral antibodies directed against tumor and histocompatibility antigens. Burdick et.al. (1973) improved the radioimmune antiglobulin test (RIAT)

and rendered it more sensitive for the detection of cell-surface bound antibodies. Consequently, Goldstein et.al. (1973) used the RIAT to demonstrate specific anti-tumor antibodies in the sera of lymphoma bearing mice. However, none of these methods were applied for the study of in situ TAIgs.

Some researchers have used protein A from S.aureus bacteria strain Cowan I, which could bind to mouse IgG2a, IgG2b, and IgG3 as well as human IgG1, IgG2 and IgG4 globulins passively bound to the surface of cells (Kronvall et.al. 1970). Brown et.al. (1977) employed ^{125}I-labelled protein A to study anti-tumor antibodies bound to cell-surface antigens. Although the latter was found to be more sensitive than the RIAT, it had the inherent disadvantage of only detecting certain immunoglobulin subclasses. The RIAT is now a widely used method for the study of in vivo bound TAIgs (Witz, 1977 and James et.al. 1979) However, other methods such as mixed hemadsorption, hemagglutination, and immuno fluorescence have also been used (Irie et.al. 1975; Witz 1977 and Haskill et.al. 1977).

Although sufficient evidence now exists indicating the in situ association of immunoglobulins with the tumor, the immunological role of these immunoglobulins and their proportional distribution on the different cells within the tumor remains to be established.

2.2.1 Tumor-associated immunoglobulins in experimental animals

The association of immunoglobulins with experimental tumors in vivo is now well documented (Witz 1977). Although some of these TAIgs have been shown to be tumor specific, others have been found to be either natural immunoglobulins or non-tumor related antibodies. For example, mice treated with bovine serum albumin (BSA) prior to the injection of tumor cells were found to have anti-BSA antibodies

on their ascitic tumor cells (Witz, 1977).

Haskill et.al. (1977), using an indirect immunofluorescence test, were able to detect IgG2a and IgG2b on tumor cells. The latter were found not to be saturated with immunoglobulins, as they absorbed additional anti-tumor antibodies. Further studies indicated that the production of these TAIgs greatly depended on thymus derived cells for all Ig classes and subclasses except IgG2b. Dorval et.al (1976) showed that various in vivo grown tumors were able to fix protein A. Tonder et.al (1976) determined the amount of IgG on 10^6 tumor cells and showed it to vary from less than 100 ng to approximately 600 ng. Only a minority of the latter was found to be bound to Fc-receptor cells within the tumor.

In another study, Ouchterlony analysis revealed the presence of antiviral IgG2a and IgG1 in tumor eluates (Goldroson and Dent, 1977). The former was detectable at all tumor growth stages studied, while the latter was only detected on tumors that were in situ for more than 60 days. IgA and IgM were also found but their presence was not correlated with tumor age. Robins (1975) was also able to detect tumor specific antibodies on an ascitic variant of rat hepatoma.

In general, current research into TAIgs in situ centers around the following: identification of the cell types within the tumor which possess or produce TAIgs; elucidation of TAIg kinetics; and determination of the in vivo and in vitro effects of TAIgs on tumor cells and on host cells.

In some tumor systems, like melanoma, the immunoglobulins have been found to be exclusively associated with the host cells (Witz, 1977). In most of the other systems, however, TAIgs have been associated with both tumor cells and host cells, some of the

latter consisting of Fc-receptor host cells (Tonder et.al., 1976 and Witz, 1977).

In vitro studies on TAIg binding kinetics showed that the surface bound immunoglobulins were in active equilibrium with the culture medium. Yeenof et.al (1976), investigating membrane bound 7S IgM on Daudi cells, discovered that within 10 hrs of adding ^{125}I-anti IgM, more than 80% of the cell bound radioactivity was shed from the cells. This took place concurrently with the loss of IgM molecules from the surface of tumor cells, probably due to internalization. Although the shed antibodies could rebind to fresh Daudi cells, some of them were found to be in the form of immune-complexes. Caspi et.al. (1976) obtained similar results with MDAYmurine tumor cells. Freshly explanted MDAY cells which were sensitive to complement mediated lysis lost this sensitivity upon short term culture at 37^{o}C. Witz and Moav (1978) detected IgG2a on SEYF-a cells, a polyoma virus-induced ascites tumor cells. The specific cytotoxic activity of tumor eluates which was highest in 11-15 day old tumor cells, decreased with the propagation time of the tumor. This could have been due to the binding of noncytotoxic antibodies or to the emergence of molecules capable of inhibiting such activity. Moreover, the tumor eluates were found to have higher binding constants than antitumor antibodies obtained from the sera of tumor bearing mice. In an extension of these studies, Moav et.al. (1978) determined two distinct populations of molecules in tumor eluates: one with high binding constant to the tumor cells, and another with a much lower binding constant to these cells. It was suggested that the former consisted mainly of antibodies to surface antigens of tumor cells.

Ran et.al (1978) showed that antilymphocyte cytotoxic antibodies were amongst the eluates of SEYF-a tumor cells. Since the titer of these antibodies in the sera of tumor bearing mice was lower than that of the tumor eluates, it was suspected that tumor cells selectively absorbed the lymphocytotoxic antibodies from the serum.

Rittenhouse et.al.(1978) also demonstrated the spontaneous release of high-molecular-weight aggregate containing IgG from the surface of Ehrlich ascites tumor cells during incubation for 1 hr in cold isotonic buffer. Wolosin and Greenberg (1979), in a competitive radio-immunoassay study, showed that several tumor lines acquired Ig rapidly between 3-18 hrs after intra-peritoneal implantation into normal syngeneic mice. The in vivo acquisition of these TAIgs was found to be a T-independent phenomenon. Moreover, since the kinetics of normal syngeneic serum binding to tumor cells in vitro matched that of TAIg in vivo, the authors deduced that the latter was due to in vivo binding of natural antibodies to tumor cells. The in vivo and in vitro effects of TAIgs are elaborated in section 2.2.3.

2.2.2 Tumor-associated immunoglobulins in cancer patients

TAIgs have also been discerned in human neoplasia (Witz, 1977). Dorsett et.al. (1975) were able to isolate tumor specific antibodies from effusions of ovarian carcinomas. Vanky et.al. (1975) found TAIgs in 18 of 44 cell suspensions (41%) prepared from surgical specimens of human tumors. Only tumor cells without TAIgs were capable of stimulating autologous lymphocytes. Infiltrating lymphoid cells were not thought to be the carriers of the TAIgs as lymphnode cells, spleen cells, and bone marrow cells seldom exhibited surface bound Igs as much as the amount found on tumor cells. Similarly, Gerstl et.al.(1977)

found membranous and cytoplasmic IgG in surgical specimens of human lung carcinoma. IgA and IgM were also detected but less frequently than IgG. Examination of Fc-receptor host cells within the tumor showed that the latter cells were unlikely carriers of the TAIg, especially that only 10% of IgA are monomeric while none of the IgM Igs have Fc-portions available for reaction. Irie et.al. (1975), using mixed hemadsorption technique, demonstrated TAIg and complement on the cell surface of human biopsies and autopsies. Gupta and Morton (1975) found specific anti-tumor antibodies in eluates of human melanoma cells which contained more IgG than IgA.

2.2.3 In vivo and in vitro effects of tumor-associated immunoglobulins

The in vivo and in vitro effects of TAIgs fluctuate between tumor growth enhancement and tumor growth inhibition (Witz, 1977). Tumor lysosomal extracts are thought to be responsible for cleaving the Fc-portion of surface-bound antibodies rendering them incapable of complement mediated lysis but capable of retaining their blocking effect (Dauphine et.al., 1974 and Keisari and Witz, 1975). Moreover, antigens released from tumor cells are capable of deterring the action of tumor specific antibodies by the formation of immune-complexes (Baldwin and Price, 1976).

The in vivo acquisition of Igs by tumor cells have been found to be inhibited by IV or IP injection of tumor antigens (Wolosin and Greenberg, 1979).

TAIgs in human tumor biopsies were not found to stimulate autologous lymphocytes, unlike biopsies that were free of TAIgs and which did stimulate lymphocytes (Vanky et. al, 1975). Moreover, TAIgs in

eluates of SEYF-a tumor cells were found to mediate complement dependent cytotoxicity of normal lymphnode cells (Ran et.al. 1978) On the other hand, TAIgs have also been found to be tumor inhibitory. A study of T-1699 murine adenocarcinoma suggested that IgG2a were active in macrophage mediated cytotoxic reactions and IgG2b in the immediate hypersensitivity reaction to T-1699 cells (Haskill et.al. 1977). Johnson, et.al. (1977c) determined that between 70,000 and 130,000 surface bound IgG1 anti-tumor antibody molecules were sufficient to cause 50% suppression of tumor growth in mice inoculated with 50,000 lymphoma tumor cells. TAIgs obtained from tumor eluates of SV40 tumors did not block in vitro lymphocyte dependent microcytotoxicity of immune lymphocytes against cultured SV40 tumor cells (Goldrosen and Dent, 1977). The antibodies in the latter eluates, however, were antiviral antibodies.

TAIgs eluted from tumor cells at low pH were incapable of rebinding to cultured cells, unlike those eluted by prolonged incubation at 37°C which retained their binding capacity (Witz, 1977). An analysis of acid eluted TAIgs revealed that they consisted of free Igs, antigen-antibody immune complexes, and degraded Igs. Tumor cells maintained in culture for 1 hr became insensitive to complement action even though 70-80% of the TAIgs were still cell-associated (Witz, 1977). It was suggested that the TAIgs were interiorized.

In conclusion, although TAIgs exist in experimental and human tumors, their distribution on the different cells within the tumor and their role in tumor inhibition or enhancement remains to be elucidated.

2.3 Practical applications of anti-tumor antibodies

Like most other fields of tumor immunology, the field of practical applications of anti-tumor antibodies has only recently emerged. Some progress has already been achieved, especially in the realm of chemotherapy and tumor diagnosis. In chemotherapy, attempts have been made to use anti-tumor antibodies as drug carriers in order to restrict damage to tumor cells and spare the normal cells. In diagnosis, anti-tumor antibodies have been used to detect both original tumors and tumor metastases.

The problems residing in the use of anti-tumor antibodies in chemotherapy included the following: the availability of biochemical sites for linking the drug to the antibody without effecting the activity of either; the binding of therapeutically effective amounts of the drug to the antibody; the delivery of the drug in active form to the tumor site; emergence of host reactions to the protein; and the emergence of resistant tumor cells (Ghose and Blair, 1978). Ghose et.al. (1976) were able to demonstrate preferential localization of chlorambucil-linked anti-tumor antibodies in the mice tumors. Mice injected with drug-linked anti-tumor antibodies exhibited a higher survival time than mice injected with either the drug or the antibody alone. These studies were extended in include 24 patients with progressive metastatic malignant melanoma, where encouraging results were also obtained. Furthermore, the preferential localization of ^{131}I-linked anti-tumor antibodies in the tumors of 2 cancer patients was demonstrated.

To date, anti-tumor antibodies have been used in attempts to improve the therapeutic effects of the following agents (Ghose and Blair, 1978): chlorambucil and Trenimon (alkylating agents);

daunorubicin, adriamycin and bleomycin (antibiotics); methotrexate (antimetabolite); phospholipases and microbial toxins (cell surface agents); and diptheria toxin and toxic plant proteins (protein-synthesis inhibitors). Although minor improvements in the specificity of these anti-tumor agents have been noted, the practical application of drug-linked anti-tumor antibodies remains very limited. The unavailability of sufficient amounts of allo or iso-anti-tumor antibodies has greatly contributed to this limitation.

Anti-tumor antibodies have also been utilized in the detection of tumor growth. Ting (1976) was able to detect anti-tumor antibodies in the sera of tumor-bearing mice at a relatively early stage. Consequently, the presence of such antibodies in human sera could be used as an early warning system of tumor growth. In another study, ^{125}I-labelled tumor-specific antibodies, isolated from mice with neuroblastoma, were found to localize preferentially in the tumor when reinjected into tumor bearing mice (Terman et.al. 1975). These labelled anti-tumor antibodies could be utilized in the detection of occult neoplasia in vivo.

2.4 Conclusion

It is apparent from what have been stated above that, while considerable progress has been made in the detection and isolation of anti-tumor antibodies, a great deal remains to be understood before they may be utilized in tumor monitoring and eradication. Progress has been and still appears to be complicated by the number of experimental tumor systems, and the various types of human cancer that exist. Whereas in one system tumor-associated Igs inhibit tumor growth, in another system they enhance it. While in one system in situ TAIgs is wholly associated with tumor, in another system they are associated with both, host and

tumor cells. Moreover, work on drug-linked anti-tumor antibodies has been hampered by the lack of sufficient amounts of antibodies and the instability of such complexes.

As mentioned, however, all classes and subclasses of antibodies have been associated with tumors, both in the sera of tumor-bearing animals and patients, and within their tumors. Current research on antitumor antibodies centres around the elution of in vivo bound TAIgs from the tumor cells; the determination of their type and specificity; the identification of the cells within the tumor; the elucidation of the in vivo mechanisms by which TAIgs enhance or inhibit growth; and more efficient utilization of anti-tumor antibodies in chemotherapy and tumor diagnosis.

3. NONSPECIFIC BINDING IN SITU

It is probable that a significant proportion of the tumor-associated-immunoglobulins are comprised of nonspecific antibodies, natural antibodies and/or immune-complexes, trapped in the tumor during their circulation through it. Certain of these non-tumor related immunoglobulins and immune complexes may bind to tumor cells and host cells inside the tumor through Fc-receptors found on these cells. Similarly, certain complement components have been detected on in vivo growing tumors. However, the in vivo role of complement in tumor immunity is only slightly understood.

3.1 Non-specifically associated antibodies and immune complexes

It is assumed that non-tumor related immunoglobulins and immune-complexes are bound to Fc-receptor bearing cells within the tumor. The existence of such cells with Fc-receptors in tumors is well documented (Witz, 1977). Kerbel et.al. (1975) exhibited a high proportion of Fc-receptor bearing cells in a variety of in vivo growing tumors. Many of these cells were found to be actively phagocytic and some were found to be of T-cell origin. Moreover, there were indications that the majority of these cells were of host origin. Tumor cells cultured in vitro over long periods of time were devoid of such cells. Similarly, Szymaniec and James (1976) showed the presence of Fc-receptor bearing cells in methyl-cholanthrene induced fibrosarcomas. The existence of these cells in tumors and their in vivo kinetics was also reported by Pross and Kerbel (1976).

Before presenting evidence on the presence of immune-complexes or non-specifically bound immunoglobulins in tumors grown in vivo, their relationship to tumor growth is briefly expounded. Immune-complexes have been found to play a significant role in blocking the anti-tumor effect of host cells (Price and Robins, 1978). Circulating immune-complexes have also been detected in the progressor sera of patients with neuroblastoma (Jose and Seshadri, 1974). The greatest blocking effect of these immune complexes was manifested at antigen-antibody equivalence. Tamerius et.al. (1975) postulated that the blocking factor noted in the sera of multiparous mice consisted largely of immune-complexes between embryonic antigens and IgG2a and IgG2b. Immune complexes were also isolated from effusions of ovarian carcinomas

(Dorsett et.al., 1975). Bowen et.al. (1975)showed that sera obtained from animals bearing small tumors (7-14 day old) were free of immune-complexes, in contrast to sera obtained from animals bearing large tumors (24-28 day old). The latter were capable of blocking the cytotoxicity of syngeneic lymphnode cells against cultured tumor cells. It is probable, therefore, that immune-complexes contribute to the escape of the tumor from cellular immune destruction.

In addition to the evidence showing the existence of Fc-receptor bearing cells in tumors and the presence of immune-complexes in the sera of tumor bearers, it is now becoming clear that immune-complexes actually bind to these cells in vivo thus probably blocking the destruction of the tumor by host cells. Braslawsky et.al. (1976a) injected mice with bovine serum albumin (BSA) or ovalbumin (OA) prior to the injection of tumor cells. Examination of the 7-9 day old ascitic tumors revealed that BSA or OA treated mice contained anti-BSA or anti-OA antibodies respectively on the freshly prepared tumor cells. The removal of phagocytic and adherent cells from the tumor cells population did not completely eliminate the in vitro binding of antibody-complexed BSA or OA to the tumor cells, indicating that tumor cells per se might be capable of binding immune-complexes. However, experiments with hybrid mice did show that the binding of immune-complexes was mostly limited to infiltrating host cells.

The in vivo binding of immune-complexes to ascitic tumors was shown to increase with time (Braslawsky et.al., 1976b). In contrast in vivo bound antibodies lost their cytotoxicity with time. Consequently, it was postulated that tumor antigens and receptors for immune-complexes

were in close proximity on the tumor cell surface, thus enabling the immune-complexes to inhibit the binding of specific cytotoxic antibodies to the antigens on tumor cells. In a later study, however, it was shown that the treatment of tumor cells by specific anti-tumor antibodies inhibited the fixation of immune-complexes by these cells (Braslawsky et.al. 1976c). It may be deduced, therefore, that should there initially be a large influx of anti-tumor antibodies into the tumor in vivo, then these antibodies could inhibit the subsequent binding of immune-complexes and prevent their detrimental blocking of cell mediated immunity. Unfortunately, spontaneous tumors do not appear to possess antigens that could evoke the early production of appreciable amounts of specific antibodies.

The nature of Fc-receptors on tumor cells per se needs to be elucidated, although there are indications that they might be produced by viruses in some tumors. Herpes simplex virus have been found to produce Fc-receptors on infected cells in vitro which could bind IgG molecules of several species (Westmoreland and Watkins, 1974). Cytomegalovirus infected fibroblasts were also found to develope Fc-receptors for IgG but not IgM or IgA, molecules (Keller et. al. 1976). It is possible, therefore, that in addition to binding to Fc-receptor bearing host cells within the tumor, immune complexes and non-tumor related immunoglobulins bind to some tumor cells via Fc-receptors produced by viruses.

3.2. Complement

Although little is known about the in vivo role of complement in tumor immunity, its association with tumor growth has been observed. Nishioka et. al. (1976) showed high levels of complement in the sera of

lung cancer patients. Further studies led these researchers to propose that the levels of complement increased in these patients in order to compensate for the decline of cell mediated immunity. Complement component (C3) has been detected on freshly prepared human tumor cells (Irie et. al. 1974; and Irie et. al., 1975), and on Erlich ascites tumor cells (Rittenhouse et. al., 1978). Experiments by Segerling et. al. (1976) on guinea pig hepatoma ascitic tumor cells suggested that the in vivo binding of complement components (C3 and C4) increased with time.

Like anti-tumor antibodies, complement may be present in the vicinity of the tumor cell membrane, but fail for one reason or another to exert its cytotoxic effect. The latter appears to depend on the growth cycle of the tumor cells, the production of anti-complementary factors and/or the secretion of extracellular enzymes by the tumor cells. Cooper et. al. (1974) showed that the complement-mediated lysis of virally induced lymphoma cells was limited to the G1 phase of the cell cycle. Complement lysis did not occur during the other phases of the cell cycle even though complement components (C5-C8) were bound throughout these phases resulting in the characteristic plasma membrane lesions. In another study, it was indicated that tumor cells might produce extra-cellular enzymes that could cleave the Fc-portion of surface-bound antibodies thus destroying their ability to bind complement (Keisari and Witz, 1975). Fridman et. al. (1974) demonstrated that sensitized lymphnode cells produced an immunoglobulin binding factor which could protect IgG sensitized erythrocytes, but not IgM sensitized erythrocytes, from complement mediated lysis. This immunoglobulin-binding-factor was shown to be distinct from other known lymphokines. It is probable, therefore, that factors produced either by tumor cells or host cells could prevent tumor-associated-immunoglobulins from binding complement and causing the lysis of tumor cells.

3.3 Conclusion

In conclusion, therefore, humoral factors including non-specific immunoglobulins, immune-complexes, and complement, could be amongst the elements present within the tumor microenvironment. Non-specifically associated immunoglobulins and immune-complexes appear to bind to Fc-receptor bearing cells thereby blocking, on the one hand, the anti-tumor action of host cells, and on the other hand, preventing anti-tumor antibodies from exerting their effect. The action of complement appears to be hindered by agents binding to or cleaving the surface-bound anti-tumor antibodies, and by phases of the cell cycle. It is hoped that further research will result in the control of these humoral factors and lead to the prevention of their detrimental effects.

4. TUMOR SPECIFIC CELLULAR IMMUNITY

Since cell-mediated immunity was found to be responsible for allograft rejections and since immunodeficient and thymectomized animals exhibited increased incidence of certain tumors, it therefore followed that cell-mediated immunity could be important in the prevention of tumor growth (immunosurveillance theory). However logical the concept of immunosurveillance may have seemed, facts do not wholly support it yet. Apart from DNA viral tumors, cellular immunity has not yet been

found to be necessary for the prevention of experimental spontaneous or virally (RNA) and chemically induced tumors. Moreover, some researchers have related the increase of lymphoreticular tumors in immunodeficient animals to the lack of T-cell defense against oncogenic viruses and not against tumors.

On the other hand, the association of T cells with certain transplantable and human tumors has been demonstrated. The methods employed for T-effector cell studies have varied from colony-inhibition and microcytotoxicity to tests with IUDR and Cr labelled tumor cell targets (Beverley, 1978). With most of these assays, clear evidence for T-cell involvement has been obtained with murine sarcoma virus tumors, TI699 mammary adenocarcinoma, human Burkitt's lymphoma and other tumors.

4.1 Tumor-specific T-cells in experimental animals

The existence of specific anti-tumor T cells in experimental tumor-bearing animals has been frequently reported (Beverly, 1978). Viral antigens were found to be the target of T cells in some of the experimental tumors. When lymphocytes, which have been primed *in vivo* with MuLV-G leukaemia cells, were stimulated with disrupted MuLV in culture, effector cells with high cytotoxicity against leukaemic cells were generated (Bruce *et.al.* 1976). Plata *et.al.* (1976) identified T cells as the cytolytic agents in a secondary mixed-leukocyte-tumor cell culture. Spleen cells obtained from regressor mice that had rejected murine sarcoma-virus induced tumors were cultured with syngeneic RB1-5 tumor cells. During 22 days of culture, T cells appeared to change from small cells to large cells and then revert back to small cells, simulating *in vivo* blastogenesis and maturation. The cytotoxicity of these T cells was found to be

independent of macrophages and non T-cells. A comparison of these T cells with T-cells obtained from allogeneic mixed-leukocyte-culture indicated that the underriding mechanism could be cell-mediated immunity against the tumor.

Holden et.al. (1976), using a Cr release assay (CRA) and growth inhibition assay (GIA) in primary murine sarcoma-virus (MSV) induced tumors, found small T cells to be active in CRA and macrophages to be active in GIA. An examination of the host-cell content of the tumors showed that 30%-40% were T cells, 50% were macrophages, and 5% were B cells. Moreover, the T cells were found to be tumor-specific, unlike the macrophages which were found to react against non cross reacting antigens. No T cells were observed in progressing MSV tumors. Similarly, in a comparison between regressing and progressing Moloney-sarcomas, Russel et. al. (1976 a & b) found more T lymphocytes in the former on all days tested post-tumor-cell injection. On day 6 of tumor growth in regressing tumors, the T cells were found to be large in size which led these researchers to propose that these cells might be indispensable for tumor regression due to their homing and direct cytotoxic properties. Moreover, inflammatory cells in progressing tumors were found to be limited to the periphery of the tumors, unlike regressing tumors whereby the inflammatory cells were found to have infiltrated the tumor mass.

Small and Trainin (1976) showed lymphocytes to be significant in a benzpyrene-induced fibrosarcoma and a spontaneously occuring carcinoma of C57BL mice, and in an MCA-induced fibrosarcoma of C3H mice. Both in vivo and in vitro experiments indicated that sensitized spleen cells consisted of two subpopulations. One comprised of large cells which enhanced tumor growth and another comprised of small cells which inhibited

tumor growth. The growth enhancing lymphocytes were always dominant when the two subpopulations of the cells were injected together. It was not clear at the time whether the two types represented different kinds of lymphocytes or the same kind but with varied degrees of maturity. In support of the latter study, Fujimoto et.al. (1976a) demonstrated that immunosuppressor T cells were a significant constituent of T cells in mice bearing an MC-induced sarcoma. The i.v. transfer of washed thymus or spleen cells from tumor-bearing animals to immune animals was found to significantly weaken their capability to reject the syngeneic tumor. Moreover, splenectomy of tumor-bearing mice or the treatment of normal syngeneic mice with anti-lymphocyte serum after tumor-cell inoculation resulted in the suppression of tumor growth. Further studies showed that these immunosuppressor T cells appeared within 24 hours after the inoculation of tumor cells and lasted until 5 days after the excision of the tumor (Fujimoto, 1976b). Although the immunosuppressor cells were observed in the thymuses, spleens, draining lymphnodes, and the bone marrow of tumor-bearing mice, they were not detected in the peripheral blood. As for the nature of these immunosuppressor T cells, they were shown to be mature cells of relatively small size. The relationship between the immunosuppressor T cells and other T cells such as effector and helper T cells remains to be established.

Haskill et.al. (1976) postulated that T cells were important in regressing TI699 mammary adenocarcinoma since the tumors became progressing after treating the mice with anti-lymphocyte-serum. Their experiments suggested that T-cells elicited a delayed hypersensitivity reaction which led to the influx of macrophages into the tumor.

4.2 Tumor-specific cell-mediated immunity in immunodeficient experimental animals

Studies on T cells in immunodeficient animals were carried out in order to establish the role of T cells in the prevention of spontaneous tumor growth and hence to examine the validity of the immunosurveillance theory. These studies indicated that while T cells were important in the prevention of DNA viral tumors, like polyoma tumors, they were hardly necessary for the prevention of other types of tumors (Stutman, 1975).

Although increased lymphoreticular neoplasma were found in nude mice, the incidence of these tumors were related more to the lack of T cell protection against viruses than tumors per se (Holland et.al., 1978) Indeed, a reduced risk of solid tumors was observed in these nude mice, though this could have been due to their short life span. Moreover, in a study of 15,700 nude mice corresponding to 5,600 mouse years, not a single incidence of a spontaneous tumor was reported (Rygaard and Povlsen, 1976). The latter authors argued convincingly that infectious disease rather than somatic mutation was the reason behind the evolution of T cells and its raison d'etre. The importance of T cells against viral oncogenesis was also emphasized by Allison et.al. (1974). However, nude mice treated with anti - lymphocyte serum in the latter study developed skin tumors earlier than nude mice that remained untreated. This led to the suggestion that a second surveillance mechanism independent of T lymphocytes might exist. Evidence for the latter has also come from other studies (Kiessling et.al. 1975; Herberman et.al. 1975; and Kiessling et.al. 1976).

Norbury and Kripke (1978) studied ultraviolet carcinogenesis in T-cell depleted mice. Although tumors were found to develop earliest in the latter mice, they grew most rapidly in mice T-cell depleted but

restored with thymus grafts. Therefore, contrary to the immunosurveillance theory, the presence of T cells proved to be stimulatory to tumor growth. In fact, it appears that the immunosurveillance theory is being replaced by the immunostimulation theory, which predicts that the growth of tumors may be due to a reduced immune response (Prehn, 1976).

4.3 Tumor-specific cell-mediated immunity in cancer patients

The role of cell-mediated tumor immunity in Man remains far from being clear. Reports indicating the presence of tumor-specific T cells in cancer patients have been contradicted by opposite reports. The confusion has been largely blamed on the absence of proper controls and lack of uniformity of assay systems used (Bean et.al. 1975).

Jondal, et. al. (1975) demonstrated the presence of tumor specific T cells in a Burkitt's lymphoma (BL) biopsy. Killer T cells from patients with infectious mononucleosis (IM) were able to recognize tumor cells recovered from the biopsy. These results led the researchers to propose that patients with BL exhibit a cell-mediated immunity and that this immunity could be strengthened by the transfer of killer cells from patients with IM. Similarly, cell-mediated anti-tumor immunity was demonstrated in the majority of 373 cancer patients (Hellstrom et.al. 1971). The observed anti-tumor effect of lymphocytes isolated from cancer patients was shown to be specific since they did not exert any effect on tumor cells obtained from different neoplasms. In contrast to these studies, Takasugi et. al. (1973) showed that lymphocytes isolated from normal individuals were as cytotoxic, if not more, as lymphocytes from cancer patients against cultured tumor cells from 7 established lines and 12 short-term cultures. The only specific cell-mediated tumor immunity observed was that by lymphocytes obtained from bladder cancer patients.

A workshop on cell-mediated cytotoxicity for bladder carcinoma carried out in 1974 at the Sloan-Kettering Institute, New York, revealed the complexities involved in the evaluation of cell-mediated immunity in cancer patients (Bean et.al. 1975). It was concluded that only a continued cooperation among the different laboratories based on uniform assays, uniform patient-control selections, and uniform statistical analyses would result in the understanding of tumor cell-mediated immunity. The intricacies of tumor cellular immunity were also discussed by Baldwin (1975) who stressed the importance of characterization and standardization of target tumor cells, tumor-associated antigens, effector cells, and microcytotoxicity assays.

4.4 Immunodepression and cancer in Man

Although increases in the incidence of certain tumors have been shown in immunodepressed patients, it is not clear yet whether immunodepression is the cause or the result of this increase in tumor growth. In a long and detailed report on immunodepression and cancer in Man and animals, Stutman (1975) demonstrated that immunodeficient individuals exhibited increased lymphoreticular malignancies and that organ transplant recipients usually developed lymphomas. On the other hand, incidence of cancer among patients with myasthenia gravis was shown to decrease after thymectomy. Cessation of immunosuppression in organ transplant patients, was found to inhibit tumor appearance (Penn and Starzl, 1973). Moreover, thirty patients with noncancerous diseases who were treated with immunosuppressives developed cancer.

Depletion of lymphocytes has been associated with advanced Hodgkin's disease and its poorer prognosis (Hansen and Good, 1974).

Furthermore, although T-cell deficiencies were noted in chronic lymphatic leukaemia, T cells isolated and concentrated from these patients exhibited normal phytohemagglutinin responses.

4.5 Conclusion

Although evidence exists for tumor specific cell-mediated immunity in experimental animals, evidence for specific cellular immunity in cancer patients remains to be established. As mentioned above, the task for determining the significance of tumor-specific cell-mediated immunity in cancer patients appears to be tedious and long, depending largely on the collaboration among the different laboratories.

Both tumor-suppressor T cells and tumor-enhancer T cells have been shown to exist in tumor-bearing experimental animals. The various mechanisms involved are not yet fully understood and may be complicated by the fact that T cells isolated from peripheral blood may not necessarily be the same as those obtained from the tumor. Haskill et.al. (1976) demonstrated that host cells isolated from the tumor were very active in colony-inhibition tests, unlike the cells isolated from spleen, lymphnodes, Peyer's patches, or blood. It was suggested that the active host cells were either sequestered by the tumor, or that these cells were produced within the tumor.

The immunosurveillance theory has been shown to be restricted to DNA viral tumors in experimental animals. In Man, however, the validity of the theory has been inconclusive. Its examination has been hampered by the combination of different kinds of immunode ficiencies at different ages under the single title of 'primary immunodeficiency', by sampling procedures and by inaccurate tumor diagnoses (Stutman, 1975).

5. TUMOR NONSPECIFIC CELLULAR IMMUNITY

Although considerable research has been carried out on the relationship between macrophages and cancer, the study of other immunologically active cells like the polymorphonuclear leukocytes (PMN) and K cells, have been relatively neglected (Sadler and Castro, 1978). Macrophages have been found to react against tumors and inhibit its growth. However, the mechanisms by which non-specific cellular immunity operates in tumor-bearers remain unknown.

5.1 Macrophages in cancer

Macrophages have been widely studied in cancer where sufficient data has been produced to indicate the association of macrophages with tumors, and its importance in tumor growth inhibition (see e.g. 'The Macrophage anc Cancer', James et.al. 1977). In general, macrophages, unlike certain types of T cells, have been found to act against the tumor rather than facilitate its growth. However, overproduction of macrophages has been shown to suppress lymphocyte production and thus possibly enhance tumor growth (Rhodes, 1977).

Miller and Feldman (1977), working with rat moloney sarcoma, demonstrated that macrophages together with antibodies could inhibit tumor growth in vivo. However, this macrophage-antibody cooperation was found to be restricted to IgG2b, while IgM and IgG1 were found to be ineffective, and IgG2a was found to intensify tumor growth. Moreover, hyperimmune peritoneal exudate macrophages were found to inhibit, and in some cases eliminate, tumor growth even without the involvement of antibodies.

Lohmann-Mathes et.al. (1979) ascribed tumor growth inhibition to macrophages which had been allowed to mature from bone marrow cells in culture. In contrast to the latter, peritoneal and spleen macrophages were found to be ineffective against tumor cells. In an extension of this work, the lytic cells were identified as non-adherent non-phagocytic promonocytes, which if permitted to mature in vitro to phagocytic macrophages, retained their lytic activity at 1:1 phagocyte target cell ratio (Domzig and Lohmann-Mathes, 1979). Similarly, nonphagocytic ADCC effector cells obtained from mammary adenocarcinoma T1699 were found to be related to macrophages (Haskill, 1977). These cells became phagocytic after an overnight incubation.

In some instances the macrophage content of the tumor has been found to be as high as 50% of the host infiltrating cells (Holden, 1976; and Russell, 1976). Macrophages were shown to be tumor inhibitors as long as the tumor-cell number did not exceed a certain threshold (Norman, 1978). Once the latter was surpassed, a complete suppression of macrophages possibly by the tumor cells, ensued. In the same study, macrophages were found to be susceptible to the ascitic fluid obtained from the tumor-bearing mice.

In conclusion, although the significance of macrophages in tumor growth inhibition is well documented, the mechanisms are not clear at the present time. However, the following mechanisms can be envisaged (Van Furth, 1977 and Shin et. al. 1978):

a) inhibition of the tumor by complement components, lymphotoxins or lysosomes that are released through bridges between the macrophage and the tumor cell.

b) suppression of tumor cells by macrophages through soluble mediators.

c) cytolosis of the tumor cells by membrane fractions of macrophages

d) release of factor-increasing-monocytopoieses(FIM) by macrophage-

tumor interaction. FIM could fail to reach the tumor due to a defect in chemotaxis.

Whatever the mechanisms involved, macrophage proliferation and its functions against the tumor seem to be dependent on tumor cell type and number (VanFurth, 1977).

5.2 Polymorphonuclear leukocytes in Cancer

Studies on the role of PMN in cancer have been hindered by the fragility of these cells and by the preoccupation of research workers with lymphocytes and macrophages. In general, however, the activity of PMN in cancer patients have been found to be depressed.

McRipley et. al. (1967) studied the phagocytic activity of peripheral blood leukocytes against E. coli, P. aurigincsa and S. albus in patients with various malignant diseases. PMN from most of these patients exhibited impaired bactericidal activity and this impairment was not correlated with gammaglobulin levels. In contrast, Kalinske and Hoeprich (1969) did not find depressed activity of PMN in patients with chromic myelocytic leukaemia or patients with chronic lymphocytic leukaemia. It was argued that this discrepancy may have been due to the fact that, in the latter study, patient-control tests were carried out simultaneously and that metamyelocytes and myelocytes, which are predominant in such patients, were considered among the PMN. Strauss et. al. (1970) demonstrated decreased phagocytic and bactericidal activity of PMN in children with acute leukaemia. Moreover, although PMN from patients in remission had normal phagocytic activity, they continued to exhibit decreased bactericidal activity. In a modified technique using autoradiography, Cline (1973) also showed PMN defects in patients with acute myelocytic leukaemia.

However, PMN have been found to respond to tumor tissue. Basophil-associated-mononuclear cells in patients with breast cancer were found to respond to autologous cancer tissue (Black and Leis, 1971). This response, together with basophil counts, was increased after mastectomy.

Hexose-monophosphate-shunt activity and nitroblue tetrazolium reduction were amongst the biochemical reactions which were incorporated in later PMN studies. Using such methods, decreased number and functional capability of PMN were found in patients with acute lymphocytic leukaemia, acute myeloblastic leukaemia, and chronic myelocytic leukaemia (Skeel et.al., 1971 and Tan et.al. 1973). However, results had to be considered carefully, as false positive and false negative reactions were observed in a study carried out by Ashburn et.al. (1973).

The cause of depressed PMN in cancer patients and its relationship to tumor growth remains unclear. Bactericidal defect of PMN in children with acute lymphocytic leukaemia was found to be due to carniospinal irradiation (Baehner et.al., 1973). In another study, PMN defects in malignant patients was observed to be independent of the cancer type, the mode of chemotherapy, or the type of infection (Pickering et.al. 1975).

5.3 Allergy and cancer

Attempts have been made to study the relationship of allergy to cancer. Ure (1969) studied 140 patients in the gynaecology wards of a large hospital and found the incidence of allergy and cancer among the patients to be mutually exclusive. Similarly, a study by Meers (1973) indicated that persons who did not have allergy were three times more likely to develop cancer than allergic persons. In contrast, Shapiro et. al. (1971) failed to observe any negative correlation between

allergy and cancer. However, they argued that the number of patients employed in such studies was too small to produce significant relationships of allergy with cancer.

Since IgE and pharmacologically active cells are important in allergy, their relationship to cancer have also been studied. Circulating IgE levels were found to be depressed in cancer patients (Jacobs et.al., 1972). Rosenbaum and Dwyer (1977) reported on the involvement of IgE and its related pharmacologically active cells in cancer. Although the overall data remained confusing, they concluded that IgE and pharmacologically active cells could be important in tumor growth inhibition and therefore deserved more attention from researchers.

5.4 Conclusion

In general, the study of tumor-nonspecific cellular immunity has been overshadowed by the study of T cells and antitumor antibodies. However, considerable data has been produced indicating the importance of macrophages in tumor growth inhibition. Reports of the other cell types including PMN, remain relatively scarce and their relationship to cancer still remains to be clarified.

6. EFFECTS OF IMMUNOSTIMULANTS IN CANCER

It is arguable that one of the most practical benefits in tumor immunology has accrued from the study of immunostimulants. Not only have they been found to prevent or inhibit immunogenic experimental tumors

(reviewed by Bast and Rapp, 1976), but encouraging results have also been obtained in certain spontaneous experimental tumors and human cancer (reviewed by Mastrangelo et. al., 1976; and Baldwin, 1978). Many parameters have been found to influence the effect of immunostimulants; these include : their mode of preparation, schedule of application, route of injection and dose. Overall optimal conditions for the application of any particular immunostimulant have not been obtained since their efficacy varies according to the tumor-system or experimental animal used. However, with respect to spontaneous tumors, the most effective results have been obtained when the immunostimulant has been either injected together with the tumor cells or injected into an already established tumor (Baldwin, 1978).

Among the immunostimulants used to date have been Corynebacterium parvum (C. parvum), Bacillus Calmette Guerin (BCG), methanol extraction residue of tubercle bacillus (MER), Staphylococcus aureus and Bordetella pertusis. There are indications that in certain cases the mode of anti-tumor action of these immunostimulants is macrophage mediated and is independent of T cells.

6.1 Corynebacterium parvum in experimental animals

In 1966, Woodruff and Boak demonstrated that the growth of a mammary adenocarcinoma tumor was inhibited when C.parvum was injected iv on days 8 and 12 after tumor-cell injection. Since then the effects of C.parvum on tumor growth in experimental animals have been widely studied and its effects on cancer patients examined. These studies have, in general, confirmed the inhibitory effects of C.parvum on experimental tumors and have suggested its therapeutic benefits in cancer patients whose tumors have been treated with surgery, chemotherapy and/or irradiation.

Some of the recent work on C. parvum included a study carried out by Pimm and Baldwin (1977) on the therapeutic effects of C. parvum in various rat tumors. The best therapeutic effect was obtained when C. parvum was injected SC together with the tumor cells. In some cases, the latter treatment not only prevented the growth of the tumor, but also evoked concomitant immunity against tumor cells injected at the same time into another part of the animal. Macrophage depletion with silica was found to abrogate the preventive action of C.parvum. Similarly, results obtained by Likhite (1974a) had shown that C. parvum was most effective in Fischer rats when injected into growing subcutaneous tumors. The inhibited tumors were found to be infiltrated mainly by macrophages. Moreover, rats which have rejected their tumors, acquired permanent specific immunity against the syngeneic tumors.

The tumor-inhibitory action of C. parvum has not been consistent because, as mentioned above, its effectiveness depended upon the tumor system or the experimental animal used. For example, successful inhibition of tumor growth by the injection of C. parvum via a certain route and at a certain time in one animal system did not necessarily imply that the result would be reproduced in another animal system. Indeed, contradictory results might be obtained in different animal systems. In one study, the administration of C. parvum was found to lead to immunological enhancement of leukaemia L120 in allogeneic mice (Berd and Mitchell, 1976). This enhancement was again found to be macrophage mediated, as the reduction of macrophages caused the reversal of tumor growth. Moreover, complement-dependent cytotoxic antibodies were found to be appreciably suppressed in mice pretreated with C. parvum. In another study, the antitumor affect of C. parvum was found to differ markedly depending on the route of its injection and the anatomical

location of the tumor (Conley and Remington, 1978). Iv or ip C. parvum treatment failed to protect mice against ependyblastoma injected intracerebrally or subcutaneously, but protected the mice against tumor-cells injected intraperitoneally.

Attempts have been made to study the serological effects of C. parvum treatment. James et.al. (1976) demonstrated that C. parvum injection into normal and tumor bearing mice resulted in the increase of certain immunoglobulin levels, especially IgG2b. The latter was found to be dependent upon the dose of C. parvum and its route of injection. Moreover, these serological changes were suppressed by the administration of gold salts which are known to inhibit macrophage function. The increase in immunoglobulin levels by C. parvum treatment was found to be rather nonspecific and, in some instances, very heterogenous (James et. al. 1977a).

There is substantial evidence to suggest that C. parvum exerts its effect by stimulating macrophages (James et. al. 1976 and Pimm and Baldwin, 1977). However, the involvement of T cells, B cells and mast cells cannot be ruled out (James et.al., 1977b; and Likhite, 1974a).

6.2. C. parvum in cancer patients

Progress towards the application of C. parvum in cancer has been hindered by the lack of controlled studies and by contradictory results obtained with the animal model, where in some cases C. parvum was found to enhance tumor growth. Nevertheless, the therapeutic significance of C. parvum has been indicated in some of the trials that have been carried out.

Israel (1976) studied the therapeutic effects of C. parvum in patients with various types of cancer including disseminated breast cancer, disseminated epidermoid bronchogenic carcinoma, and oat cell

bronchogenic carcinoma. In the majority of cases, C. parvum administration was found to act synergistically with chemotherapy, resulting in improved prognosis. Although C. parvum treated and nontreated patients received the same overall dosage of drugs, those on immunotherapy were able to receive the drugs less frequently but at a higher dose each time. Consequently, it was not possible to conclude whether the improved prognosis was due to the latter differences in chemotherapy regimes or to immunological effects.

James et. al. (1975) studied the serological changes in cancer patients treated with C. parvum, where an overall increase of immunoglobulin levels, especially IgG, was obtained. It was postulated that the administration of C. parvum could inhibit the action of blocking antibodies by enhancing the production of complement-fixing antibodies.

Hirshaut et. al. (1976) attempted to evaluate the immunological capacity of C. parvum by investigating its route of injection, dose, schedule and toxicity. Their preliminary results showed that subcutaneous C. parvum administration was the least toxic.

The mechanisms behind C. parvum action in cancer are still far from clear. However, as in the animal model, the beneficial role of macrophages is suspected. It is thought that C. parvum could activate macrophages which would recognize cells that have lost their contact inhibition properties (Israel, 1976). Further support for the involvement of macrophages, as well as cytotoxic antibodies, is indicated by the results of Biran et. al. (1976) which show that C. parvum administration results in increased complement consumption. Moreover, C. parvum might decrease drug metabolism thus modifying the functions of chemotherapy (Soyka et. al. 1976) The enhancement or inhibition of chemotherapy by the latter could depend on the time of C. parvum administration.

6.3 Bacillus Calmette Guerin

With respect to BCG, considerable advance has been made in immunotherapeutic research. Its effects in cancer patients have been thoroughly investigated where BCG, either alone or with chemotherapy, was found to produce improved prognosis (Mastrangelo et. al 1976).

The immunotherapeutic effects of BCG have been observed in experimental tumors (Bast and Rapp, 1976). Like C. parvum, the action of BCG has been found to vary with the experimental tumor system used, which has hindered efforts to determine the optimal conditions for its administration. In some spontaneous tumors, however, optimal results with BCG have been obtained when it was administered together with the tumor cells (contact therapy) (Baldwin et. al. 1976). An intact immune system was not found to be necessary in order for the BCG to exert this effect, as similar results were obtained in nude mice. The therapeutic effects of contact therapy with BCG were also demonstrated by Sparks et. al. (1974). Preoperative intratumor injection of BCG or its postoperative SC injection was found to improve the prognosis of tumor bearing mice, and in some cases, even cure them.

There are indications that the mechanisms of anti-tumor action of BCG are similar to those of C. parvum. Boyle et. al. (1976) detected complement dependent cytotoxic anti-tumor antibodies in the sera of guinea pigs cured of their tumors by BCG treatment. Preston et. al. (1971) found that in vitro stimulation of lymphocytes with tuberculin resulted in the production of macrophage aggregating or migration inhibiting factors. It is therefore assumed that BCG functions

by stimulating lymphocytes which then activate macrophages or enhance the production of cytotoxic anti-tumor antibodies through lymphoKines and B cell - T cell cooperation respectively.

Many trials have been carried out with BCG in cancer patients where some encouraging results have been obtained. One such trial was conducted by Torisu et. al. (1976) who studied the effects of active immunotherapy with living BCG in 98 cancer patients with various types of cancer. A strong correlation was found between intradermal BCG administration and improved prognosis. Moreover, their results supported an immunological basis for the latter, as the effectiveness of BCG was found to be related to immune-responsiveness. Immunocompetent patients were found to respond much better to BCG therapy than patients with suppressed immunity.

The effectiveness of BCG therapy was also demonstrated by Gutterman et. al. (1976) in patients with malignant melanoma, colorectal carcinoma, and disseminated breast cancer. BCG administration by scarification was found to improve survival in melanoma patients when applied alone, and in patients with breast cancer and colorectal carcinoma when applied with chemotherapy. However, the significance of the results obtained in this study was limited by the fact that controls consisted of historical cases. In another study, postoperative lung cancer patients treated with intra-pleural BCG exhibited improved recovery (McKneally et. al., 1976). However, this effectiveness of BCG was found to be limited to patients who have had a limited tumor burden. On the other hand, no positive effects of BCG were obtained in a study carried out by Pinsky et. al. (1976) on patients with stage II melanoma. This result was explained by some as being due to improper application of BCG vis a vis the amount administered and its anatomical location.

In conclusion, it can be said that the fulfilment of the following requirements might lead to a better response to BCG therapy (Bast et. al. 1974):

a - the injection of the proper number of organisms

b - the utilization of the proper substrain

c - the achievement of close proximity with tumor cells

d - the ability of the host to develop an immune response to tumor antigens.

6.4 Other immunostimulants

Some of the other immunostimulants that were studied include MER, S.aureus, statolon, and B. pertusis. Weiss et. al. (1976) studied the effect of the MER fraction of tubercle bacilli on the in vitro cytotoxicity of the RES against neoplastic cells. The ip injection of MER into mice once or twice before the injection of tumor cells was found to stimulate the lymphoid cells both specifically and nonspecifically against the tumor cells. It was postulated that there was more to the mechanism of the stimulation than just an occasional cross-reactivity between MER and the tumor cells, or the destruction of tumor cells as bystanders. In an extension of these studies, results by Kedar et. al (1978) demonstrated similar effects of MER when administered to lymphoid cells in vitro. However, as occasionally obtained in the in vivo experiment, this effect was found to be dose dependent since higher quantities of MER produced suppression of lymphoid cells. Fractionation studies showed that cells stimulated by MER belonged to the non-adherent cell population.

Chirigos and Theodore (1976) studied the effect of S. aureus membranes (SAM) on leukaemia in mice. The id administration of SAM after

the initiation of chemotherapy was found to markedly improve the recovery of the mice. SAM application was thought to reverse the immunosuppression caused by chemotherapy and thus enable the immune system to eradicate the residual tumor cells. The interesting result obtained in this study was that the effects of SAM were independent of the dose used or its time of administration.

The immunostimulatory effects of double stranded RNA (dsRNA) obtained from statolon, which is a lyophilized medium from cultures of mycophage infected P. stoloniferum, was studied by Marx and Wheelock (1976). Leukemogenesis was suppressed in 50% of mice treated with the dsRNA iv on day 3 after Friend Leukaemia virus injection.

A study by Likhite (1974b) showed that the SC injection of killed B. pertusis mixed with tumor cells produced a specific and a lasting rejection of tumor growth. Ip injection of the killed organisms either alone or mixed with killed tumor cells conferred no such protection on the mice.

6.5 Conclusion

The therapeutic implications of immunostimulants in cancer appear to be promising. Considerable evidence suggests an immunological basis for the anti-tumor function of these immunostimulants, which if understood could lead to the cure and even the prevention of cancer. Therefore, consistent research on the mechanisms of action of the above mentioned and other immunostimulants seems to be warranted.

7. PROSPECTS OF TUMOR IMMUNOLOGY

It is apparent from what has been mentioned so far that considerable data has been produced over the past two decades to justify continued research in tumor immunology. The association of host cells and anti-tumor antibodies with tumor growth has been proved and the influence of immunostimulants on this association has been demonstrated. Further research ought to be carried out in order to obtain a greater understanding of this tumor-host association. Moreover, methods have to be devised in order to utilize tumor-growth suppressor host cells and antibodies, and eliminate tumor-growth enhancer host cells and antibodies. It is arguable that research in tumor immunology will contribute a great deal towards not only the ultimate prevention and control of tumor growth, but also towards a greater comprehension of normal and pathological immune mechanisms. In the near future, however, two main avenues appear to be open for the practical application of tumor immunology; namely, immuno-diagnosis and immunotherapy.

7.1 Tumor immunodiagnosis

The aim of tumor immunodiagnosis has been the utilization of cellular and humoral anti-tumor immunity for the early detection of tumor growth and for the assessment of post-operative and post-chemotherapy prognosis.

Preliminary work in immunodiagnosis involved the use of *in vitro* cell-mediated cytotoxicity in the evaluation of patients' cell-mediated immunity against the tumor. However, progress in the latter was hampered by the inconsistent and nonspecific behaviour of lymphocytes against tumor cells *in vitro*. Moreover, this was further complicated by the fact that the antigenic expression of a particular tumor differed according to whether it was obtained from a short term cultured, long term cultured, or an *in vivo* grown tumor. It became clear, therefore, that for greater specificity in cancer

immunodiagnosis, a technique had to be devised which could assess the reactivity of host cells against tumor antigens. The latter could be extracted and stored, and thus supply a source of constant antigenicity.

Extensive work is being conducted on the characterization and utilization of carcinoembryonic and alphafoetoprotein antigens (Shuster et. al. 1978). Although the use of these two tumor-associated antigens is hindered by their presence in the sera of some normal individuals, their purification and identification could contribute appreciably to the early detection of tumor growth. On the other hand, efforts are being made to extract antigens from the surface of tumor cells and use them in tumor immunodiagnosis.

An assay that could specifically detect anti-tumor immunity has been the leucocyte adherence inhibition (LAI) test (Maluish and Halliday, 1974; and Marti and Thomson, 1976). It was based on the observations that normal leucocytes adhere to glass and that this adherence is inhibited if sensitized leucocytes are exposed to the autologous antigens. The LAI assay has been declared by some investigators to be sufficiently sensitive to differentiate between various tumors and to assess the progress of tumor growth (Rutherford et. al. 1977; and Flores et. al., 1977). It was concluded by Flores et. al. (1977) that the inhibition of leucocyte adherence was caused through cytophylic anti-tumor antibodies attached to the leucocytes. However, this should have been supported by demonstrating consistent LAI of normal leucocytes to which anti-tumor IgG had been bound. Moreover, the effect of antibody elution from the surface of sensitized leucocytes on LAI test was not investigated. Nevertheless, the LAI has been considerably improved and the prospects for its continued use in immunodiagnosis appear encouraging (Shuster et. al. 1978).

Other methods for tumor immunodiagnosis have included skin sensitization to tumor antigens. Char et. al. (1974) studied cutaneous delayed hypersensitivity responses to a soluble melanoma antigen in thirty-two patients with ocular melanoma.

It was found that this assay was capable of assisting in the diagnosis of ocular melanoma. Kopersztych et. al. (1976) examined cell mediated immunity in patients with carcinoma by using skin tests with ubiquitous antigens, by determining the T & B cell numbers in peripheral blood and by the response of lymphocytes to phytoheamaglutinin. By these three criteria they were able to distinguish disseminated disease from localized disease.

Anti-tumor antibodies have also been used in tumor immunodiagnosis (Ting, 1976; and Terman et. al., 1975). However, efficient utilization of this approach awaits the development of more refined techniques and a greater understanding of the in vivo mechanisms of anti-tumor antibodies.

7.2 Tumor immunotherapy

Knowledge obtained from tumor immunology research can also be practically applied in the field of cancer immunotherapy. Indeed, attempts at immunotherapy in cancer have been made for many years. Although some of these attempts have been successful, the practice of tumor immunotherapy remains largely empirical.

The rationale behind tumor-immunotherapy has been the demonstration of the existence of tumor antigens, the correlation of immunodefficiency with poor prognosis, and the relative improvement of prognosis by immunostimulation(Gutterman et. al. 1978). Tumor immunotherapy has consisted of six approaches; namely; systemic active nonspecific immunotherapy; systemic active specific immunotherapy; adoptive immunotherapy; passive immunotherapy; local immunotherapy and combined immunotherapy (immunotherapy plus surgery, chemotherapy and/or irradiation). Of these, local and combined immunotherapy have been the most successful (especially

TABLE 1 SOME POSSIBLE EFFECTS OF ANTI-TUMOR ANTIBODIES IN VIVO

A - OF ADVANTAGE TO THE HOST

1. Direct cytotoxic effect on tumor cells.
2. Coat tumor cells rendering them susceptible to phagocytosis or K cell cytolysis.
3. Activation of the complement system resulting in the generation of
   a) Kinins and anaphylatoxins which increase vascular permeability and infiltration of PMN.
   b) C3b and C4b which promote localization of various host effector cells.

B - OF ADVANTAGE TO THE TUMOR

1. Coat tumor cells rendering them resistant to attack by macrophages, K cells or antibody.
2. Modulate tumor antigens so rendering tumor less susceptible to antibody or cell mediated lysis.
3. Interact with soluble tumor antigens to form soluble complexes which inhibit attack by macrophages, K cells and T cells.

when administered in conjunction with immunostimulants). Active immunotherapy by Vibrio cholera neuraminidase treated tumor cells was found to produce tumor regression in mice when combined with surgery (Rios and Simmons, 1976). Treatment of tumor-bearing mice with V. chdera neuraminidase treated cells and BCG after chemotherapy was found to improve the survival of the mice (Cantrell et. al. 1976). Progress of immunotherapy in human cancer has also been noted in leukaemia and solid tumors (Freeman, 1978; and Gutterman et. al 1978). A potential goal of leukaemia-immunotherapy includes the in vitro sensitization of lymphocytes obtained from patients in remission, with the aim of reinjecting these cells to the same patients on relapse.

Carter (1976) outlined the difficulties encountered in immunotherapy and expressed the dire need for meticulous classification of the cancer type under study and the proper randomization of patients. Immunologists were advised to follow the consecutive phases applied in the evaluation of new drugs. These included an assessment of toxicity in phase I, a limited examination of clinical activity in phase II, and, if relevant, a large scale clinical investigation in phase III. Passive humoral immunotherapy was discouraged because of fears of tumour growth enhancement.

In conclusion, it is agreed that the practical prospects of tumor immunotherapy for the near future lie in the combined modality approach. Extensive efforts are needed to evaluate the significance of the other above mentioned approaches, as well as other immunotherapeutic modalities. The latter include the utilization of transfer-factor, immune RNA, thymic hormones, and the ultimate use of immunoprophylaxis with specific tumor antigens. Finally, increased used of antitumor antibodies in cancer chemotherapy can be envisaged. Such antibody-conjugated drugs have been shown to be more efficient in combating tumors than antibody or drug alone (Ghose and Blair, 1978; and Hurwitz et. al. 1979)

8. PURPOSE OF STUDY

As stated previously, extensive research on tumor bearing animals and cancer patients has shown that circulating anti-tumor antibodies behave in two ways. On the one hand, circulating anti-tumor antibodies have been found to produce *in vitro* and *in vivo* blocking of anti-tumor action of host cells (Tamerius *et. al.* 1975; Harris *et. al.* 1978; and Heidrick *et. al.* 1978), and on the other hand, they have been found to mediate or assist the anti-tumor action of host cells (Johnson *et. al.*, 1977 a & b; and Miller and Feldman, 1977). The extent to which each class or subclass of anti-tumor antibodies contributed to tumor growth enhancement or inhibition varied with the animal strain, type of tumor, and the extent of tumor growth. Moreover, the quality and quantity of anti-tumor antibodies has been shown to be influenced by various treatments such as BCG or *C. parvum* administration (James *et. al.*, 1975, 1976 and 1977a; and Boyle *et. al.*, 1973), and T-cell deprivation (James *et. al.*, 1977b). The extensive work on circulating anti-tumor antibodies revealed their importance in the tumor-host relationship and paved the way for further studies, especially in the use of these antibodies in chemotherapy and diagnosis. The bisided effects of antibodies in tumor-bearing hosts are summarized in Table 1.

Although research on circulating anti-tumor antibodies is continuing, appreciable attention has lately been diverted towards the study of *in situ* tumor associated immunoglobulins (TAIgs). It was realized that the study of TAIgs would result in a greater understanding of the role of anti-tumor antibodies *in vivo*. Initial studies on TAIgs were concentrated almost entirely on experimental ascitic tumors, principally because of the

relative ease by which these cells could be recovered from the animal host. These studies showed that TAIgs existed on the surface of *in vivo* grown tumor cells and that the quantity and quality of these TAIgs varied with the type of tumor, age of the tumor, and the immunological state of the host (Witz, 1977). Similar studies on solid tumors were less frequent because relevant assays to recover the tumor cells and examine their TAIg content were not available.

In view of the significant role that *in situ* TAIgs might play in solid tumors, it was the purpose of this study to develop sensitive techniques for the detection of TAIgs in solid tumors and its utilization in various studies on *in vivo* bound TAIgs. The latter were to include the following :

a - a comparison of TAIg binding in different types of tumors

b - a study of the kinetics of TAIg binding.

c - an assessment of the influence of *C. parvum* on the quantity and quality of TAIg

d - an examination of the mechanisms involved in TAIg binding.

9. APPROACH USED

9.1 Development of an indirect radio-immune-antiglobulin test (RIAT)

An indirect radio-immune-antiglobulin test which had been used for the study of circulating anti-tumor antibodies in this laboratory, was modified and adopted for the study of TAIgs on freshly excised tumor cells. It consisted of the following steps: incubating the tumor cells

prepared from a freshly excised tumor with rabbit-anti mouse immunoglobulin, in the wells of a plastic plate for an hour; washing the cells several times with 5-10% FCS ; incubating the cells with ^{125}I-goat-anti-rabbit IgG; washing the cells again for several times, and determining the counts.

One of the difficulties which had to be overcome was the preparation of tumor-cell suspensions without the use of enzymes, since the latter were known to remove surface bound immunoglobulins. This was achieved by the mechanical preparation of tumor cells which involved gently teasing tumor pieces with a pair of sterile syringe needles. Although the viability of tumor cells prepared in this manner was low, the physical shape of the cells remained intact and later studies showed that the presence of surface bound TAIgs bore no relationship to the viability of the cells.

Another factor which had to be considered was the possible elution of TAIgs from the surface of tumor cells. This was avoided by performing the assay immediately after tumor excision, and at a temperature of between 0 to 4 degrees centigrade.

Nonspecific binding of reagents to plastic was a third problem which had to be solved before the optimum conditions of the assay could be determined. A series of precoating experiments showed that foetal calf serum was most suitable for inhibiting the nonspecific binding to plastic, though this could not be prevented entirely.

Binding of reagents to tumor cells had been found to vary positively with reagent concentration and incubation time (Sparks et. al., 1969 Burdick et. al., 1973; and Brown et. al. 1977). Although an optimum incubation time could not be obtained, 80% of the reagent was shown to bind within one hour of incubation. Similarly, reagent binding in our assay was found to vary positively with reagent concentration and incubation time.

The indirect plate method proved to be sufficiently sensitive to detect TAIgs on freshly excised cells. However, it was decided to develop a tube method which could facilitate the technical aspect of the assay and improve its specificity. Therefore, a series of comparative experiments between the plate and tube methods were conducted. Similar results were obtained by both methods; however, the tube method was chosen for the application of the indirect RIAT because of its technical feasibility.

9.2 Application of the indirect RIAT

9.2.1 The response to CCH1 in nontreated and C. parvum treated mice; Preliminary studies on TAI and its in vivo kinetics were carried out on a chemically induced tumor (CCH1) which was known to be strongly immunogenic (James et. al., 1979). The variation of in vivo TAIg binding with age and size of CCH1 tumors was studied. Moreover, since C. parvum had been found to influence the humoral immune response of tumor bearing animals, its effect on in vivo TAIg binding to CCH1 tumors was also studied.

9.2.2 The response to T3 in nontreated and C. parvum treated mice: In order to determine whether or not in vivo TAIg binding depended on the type of tumor, the response to T3 (a less immunogenic tumor than CCH1, was studied. The variation of in vivo TAIg binding with age and size of T3 tumors was examined, and the influence of C. parvum on this variation was evaluated.

9.2.3 The response to CCH1 and T3 tumors: Although the response to T3 tumors was found to be weaker than the response observed against CCH1 tumors, further experiments were conducted in order to evaluate the significance of this difference in response. Hence, the variation of

in vivo TAIg binding with age and size of both tumors were examined simultaneously.

9.2.4 The response to CCH1 excised and cultured tumor cells : There have been reports of different immunological responses to tumors induced by freshly excised and cultured tumor cells (Moore, 1977; Pross et. al., 1976; and James et. al. 1979). In order to determine whether the two forms of tumor cells would elicit different in vivo TAIg binding, the response to CCH1 excised and cultured tumor cells was compared.

9.2.5 The response to different doses of CCH1 tumor cells, and to pronase prepared versus mechanically prepared CCH1 tumor cells : During the preliminary studies, it was observed that in vivo TAIg binding in nontreated tumor-bearing mice tended to increase with size of tumors. Consequently, the response to different doses of tumor cells was compared in order to determine whether TAIg binding was related to the size of tumors. Moreover, in order to evaluate further the kinetics of TAIg binding, the response to pronase prepared cells and mechanically prepared cells was compared. It was known that the two forms of tumor cells would differ in their host cell content and thus could elicit different responses.

9.3 Development of the direct RIAT

The indirect RIAT, though able to detect differences in in vivo TAIg binding among various tumors, lacked sufficient sensitivity and was laborious. Therefore, a direct RIAT was developed which could provide a better assessment of the kinetics of TAIg binding and its in vivo mechanisms.

The parameters which were specified in section 9.1, were again examined for the development of the direct RIAT. Similar to the indirect RIAT, reagent binding to tumor cells in the direct RIAT was found to vary

directly with reagent concentration, incubation time, and number of tumor cells. The specificity of the RAMIg binding to tumor cells and its binding to the latter via its Fc-portion were also assessed.

9.4 The application of the direct RIAT

9.4.1 The response to various types of tumor cells: In order to confirm earlier results obtained by the indirect RIAT, comparative studies between CCH1 and T3 tumors and between CCH1 excised and cultured tumor cells were repeated. The differences observed between these tumors using the indirect method were again noted using the direct RIAT.

9.4.2 The response to tumor cells injected into opposite limbs in the same mouse: Tumors grown on opposite limbs of the same mouse were compared in order to establish whether the response observed in different mice would be reproduced in the same mouse. The different responses to different types of tumors noted previously were again observed in the same mouse.

9.4.3 The influence of C. parvum on the response to CCH1 tumors: Additional studies were carried out to examine the effect of C. parvum on the in vivo TAIg binding to CCH1 tumor cells. While ip injection of C. parvum on day 3 after tumor cell injection was found to increase TAIg binding, no effects were observed when C.parvum was injected SC on day 3, or intratumor on day 8 after tumor cell injection. It should be mentioned, however, that the former experiment was conducted on tumors grown in different mice, while the latter two experiments were conducted on tumors grown in the same mouse.

9.5 Subclass studies

The studies mentioned above provided a quantitative assessment of TAIg in solid tumors. However, a more thorough understanding of TAIgs in solid tumors required a qualitative assessment of the surface bound TAIgs. This was deemed to be necessary especially when it was noted that C. parvum treated tumors, although smaller in size than nontreated tumors, possessed higher quantities of TAIgs. It was postulated that the latter two types of tumors could differ in their quality of TAIg in addition to their observed quantity differences. Preliminary studies were initiated to study cell-surface bound IgG1, IgG2a, IgG2b, IgG3, IgA and IgM, in C.parvum treated and nontreated CCH1 tumors.

9.6 Studies on the mechanism of in vivo TAIg binding

Finally, an investigation was made into the mechanism of TAIg binding. The studies included the effects of T-cell deprivation (in thymectomized mice and nude mice), whole body irradiation, and gold salts administration. Antibody responses in B mice have been shown to be considerably reduced (Haskill et. al., 1977). Sublethal irradiation of mice has been found to reduce the in vivo coating of tumor cells (Dorval et. al., 1976). Similarly, administration of gold salts have been observed to influence serological changes in tumor bearing mice (James et. al., 1976).

MATERIALS AND METHODS

1. ANIMALS

a) MICE

Male and female mice, 8-12 weeks old, of the inbred CBA strain were used. They were bred in our department from stock mice originally obtained from the MRC Laboratory Animal Centre, Carshalton, England. The mice were housed in plastic boxes (5-10 a box) lined with wood shavings. A constant temperature of 22°C was maintained in the room with a continuous air exchange to ensure adequate ventilation. Mice were fed with mouse cake ad libitum (McGregor & Co (Leith) Ltd., Edinburgh).

Nude mice and their litter-mates were obtained from Moredun Institute, Edinburgh and maintained in germ free conditions. Details on the precise origin of these mice was not available but some of them had recently been mated with CBA mice.

B mice consisted of thymectomized and X-irradiated CBA mice. They were thymectomized at the age of 4-6 weeks, followed ten days later by whole body X-irradiation (850 rad) with thorax shielding. The mice were then allowed to recover over a period of several weeks. Controls included non-treated and sham-thymectomized mice. T cell deprivation was assessed by the visual examination of the absence of the thymus and by the antibody response to SRBC.

b) RABBITS

Adult New Zealand white female rabbits were used. They were kept in solitary confinement in metal cages and fed on a diet of rabbit pellets supplemented with greens

c) GOATS

They were maintained under standard conditions at the University Centre for Laboratory Animals (The Bush, Willow Bridge, Midlothian)

2. TISSUE CULTURE

CCH1 21 tissue culture cells were maintained in RPMI-1640 medium buffered with 20mM HEPES and supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 ml glutamine, 100u/ml penicillin and 100 Mg/ml streptomycin.

The CCH1 tumor cells were originally induced in 8-10 week old CBA mice in our laboratory by the intramuscular (IM) injection of methyl-cholanthrene (0.5 mg) in trioctanoin(0.2 ml). They were propagated in vivo by SC injection into the limbs of mice and in vitro by tissue culture. Their passage number varied from 21-27. T3 tumors have been obtained after injection of CBA mice with syngeneic embryo cells which had undergone spontaneous transformation in vitro. Information regarding the immunogenicity and TD50 of the tumor cells is listed in table 2.

3. TUMOR CELL PREPARATION FOR in vivo TUMOR PROPAGATION

Tumor growth was produced by transplanting 1×10^6 tumor cells SC into the limbs of syngeneic mice. The tumor cells used were obtained in the following manner :

A tumor was asceptically excised and put in a sterile universal tube containing a few mls. of Dulbecco saline placed on ice. After cutting the tumor into small pieces using a pair of sterile syringe needles, it was incubated with 10 mls of pronase (pH 7.2, 2.5 mg/ml in Dulbecco RPMI buffer) at 37°C for ½ hour with occasional gentle shaking. The

supernatant was decanted, the tumor further cut into smaller pieces and reincubated with 10 mls pronase, 0.5 ml DNA ase (20% $\frac{W}{V}$) for 5 minutes with occasional shaking. The cellular suspension was then collected in another sterile universal tube through a sterile metal gauze screen filter and left on ice. The process of incubation and the collection of the cellular suspension was repeated twice or more, depending on the number of cells required. The cells were then washed three times with Dulbecco saline (224 g, 7.5 min.; MSE, England), counted, and diluted to 1×10^6 cells/ml.

4. TUMOR CELL PREPARATION FOR THE ANTIGLOBULIN ASSAY

On the day of the test, 3-5 mice/group were sacrificed by ether inhalation. Their tumor sizes were measured(average of 2 perpendicular diameters), excised, and collected in universal tubes containing Dulbecco saline. The tumors were then cut into smaller pieces using a pair of sterile syringe needles and slowly stirred in a beaker containing complete RPMI for about 10 min. The supernatant was then transferred into a test tube and the debris allowed to settle. The clear cellular suspension was then transferred by a pasteur pipette into a universal centrifuge tube, washed three times with Dulbecco saline (224 g, 7.5 min.; MSE, England), resuspended in 1 ml complete RPMI, counted and diluted to 5×10^5 cells/ml. The viability of the cells, as assessed by trypan blue dye exclusion, varied from 10-50% with no apparent change in the morphology of dead cells. Blood was also collected from the mice and the sera separated and stored at -20°C. All procedures were carried out at 4°C.

TABLE 2 SOME CHARACTERISTICS OF THE SYNGENEIC TUMORS USED IN THE PRESENT STUDY

Tumor designations	Description	Generation Nos.	How induced	Immunogenicity[+]	TD50[X]
CCH1	Fibrosarcoma	21-27	3 methylcholan-threne	10^5 - 10^6	27
CCH5	Fibrosarcoma	3	3 methylcholan-threne	10^5	830
T3	Fibrosarcoma	0-1	By transplantation of embryo cells spontaneously transformed in vitro	10^4	57

+ The challenge dose against which the preinjection of 10^6 irradiated tumor cells 2 weeks earlier affects complete protection.

X The number of tumor cells which when injected s.c. gives rise to tumors in 50% of the mice.

5. ANTISERA

a) RABBIT-ANTI-MOUSE IMMUNOGLOBULIN (RAM-Ig)

(1) $RAM\text{-}Ig_{(1)}$: This antiserum was used in the initial experiments for the development of the indirect radioimmune-antiglobulin test (RIAT). It was prepared by the IM injection of mouse IgG (1mg/ml) plus 1 ml of freund's complete adjuvant (FCA) into rabbits on day 0, followed by a similar injection on day 22 and a third intra-peritoneal (IP) injection with alum on day 42. 50 ml bleeds were obtained when required from day 50 onwards, the final bleed being on day 125. The mouse IgG used had been obtained by precipitating 7S fraction of normal mouse serum twice with 28% Na_2SO_4, dissolving in 10mM PO_4 buffer (PH 7.9), and then dialyzing overnight against PBS.

(2) $RAM\text{-}Ig_{(2)}$: This antiserum was used in the remaining indirect RIAT assays and was obtained as follows: On day 0, 5 ml of rabbit blood was collected and the erythrocytes (RBC) separated and washed three times with PBS. 3.0×10^8 RBC were injected ip into 12 CBA mice. Six mice were sacrificed on day 6 and bled out. The serum was separated and inactivated by incubation at $56^{\circ}C$ for 30 mins. The pooled serum from the 6 mice was incubated for 30 mins with freshly prepared rabbit RBC at $37^{\circ}C$ (approximately 3 ml of serum with 1 ml packed RBC). The RBC's were then washed, mixed with 1 ml FCA and injected back to the rabbits IP (for anti-IgM response). The same procedure as day 6 was carried out on day 12 on the remaining 6 mice (for anti-IgG response). Six more mice were injected with fresh RBC on day 21 and on day 28 the same procedure

as day 6 was again carried out (for boosting). On day 60, the rabbits were bled out after the presence of an adequate anti-mouse Ig response had been confirmed by immunoelectrophoretic and gel diffusion plates.

(3) RAM-$Ig_{(3)}$: This antiserum was purchased from Dako-Patts, Denmark and was used in the direct RIAT.

(4) RAM-IgG_1, G_2a, G_2b, G_3, A and M were purchased from Bionetics, Kensington, Md.

(b) NORMAL RABBIT SERUM (NRS)

(1) $NRS_{(1)}$: The normal rabbit serum, used in the initial experiments with the indirect RIAT, was a prebleed from the rabbit in which the RAM-$Ig_{(1)}$ was raised.

(2) $NRS_{(2)}$: Likewise this NRS was a prebleed from the rabbits in which the RAM-$Ig_{(2)}$ was raised.

(c) GOAT-ANTI-RABBIT Ig (GAR-Ig)

(1) GAR-$Ig_{(1)}$: GAR-$Ig_{(1)}$ had been prepared earlier by injecting goat with 10mg of a rabbit IgG preparation in complete FCA once a month for a total of 4 consecutive months, followed by a fifth injection given 2 months after the fourth. The rabbit IgG had been prepared

by the Batch chromatography on DEAE 52 cellulose of 50 ml of NRS. The goat bleeds were pooled and the IgG prepared by Na_2SO_4 salt precipitation and Batch DEAE 52 separation. The final concentration was 1 gram per cent.

(2) GAR-$Ig_{(2)}$: This antirabbit antiserum consisted of acid-eluted GAR-Ig prepared by the following: 1g of gel (Sepharose, Pharmacia Fine Chemicals, England) was swollen for 15 minutes at room temperature in 200ml of 0.001M Hcl, filtered through a sintered glass filter and washed with the same amount of 0.001M Hcl. Rabbit IgG (10mg/gel) was dissolved in 0.1M borate buffer containing 1M NaCl, PH 8, added to the gel and the contents mixed by slow stirring for 2 hours at RT. The unbound protein was removed by washing the gel on a sintered glass filter with the borate buffer. The remaining active groups on the gel was blocked by reacting for 2 hours with 1M ethanolamine at PH 8, (approximately 2 gel volumes of ethanolamine were used and the gel was stirred slowly throughout the blocking procedure). The gel was then washed in turn with 0.1M acetate buffer PH 4 containing 1M NaCl and 0.1M borate buffer PH 8 containing 1M NaCl and poured into a 10ml syringe barrel. The GAR antiserum was dialyzed against the borate buffer and applied to the column at $4^{\circ}C$ and allowed to remain in contact with it for at least ½ hour. The unbound protein was eluted with the above borate by washing with 2 or more column volumes. The specifically absorbed antibody was eluted with 0.2M glycine HCL buffer, PH 2.8, containing 0.5M NaCl. The PH of the effluent was adjusted to 7 by collecting 3 ml fractions in 1 ml of 2M Tris HCl buffer, pH 7.8. The protein containing fractions were pooled, dialyzed and concentrated. The strengths and concentrations of the GAR-Ig thus prepared are listed in Table 3.

TABLE 3 THE STRENGTHS AND CONCENTRATIONS OF THE GAR Ig ACID ELUATES

Source	Final conc.	Strength
GAR Ig A/S 2/4	0.43 g %	Fairly strong line to 1:8 Dil NRS + NRIgG
As above	0.32 g %	1/4 dil
GAR Ig prep. 238	0.42 g %	1/8 dil

(3) Swine anti-rabbit Ig was purchased from DAKO immunoglobulins, Denmark.

6. IODINATION

RAM-$Ig_{(3)}$: To 0.5ml-1ml of the protein (6mg/ml), 100 µl of 0.5M phosphate buffer, PH7.6 was added followed by 1mCi of ^{125}I (Amersham, England), and 100 µl of chloramine-T (BDH, England), with the amount of Chloramine-T being $^{1}/_{5}$ that of the protein. After 2 minutes stirring, the reaction was halted by the gentle addition of 100 µl of $Na_2S_2O_5$ (BDH, England) with the amount of $Na_2S_2O_5$ being twice that of the Chlor. T. The labelled protein was then passed through Sephadex-G25 (column dimensions: 15 cm long of approx 1.5 cm diameter), (Pharmacia Fine Chemicals, England), and collected in glass tubes in 3ml amounts. 4 µl amounts from each of the latter tubes were counted and the tubes correlating with the first peak utilized. The protein solution was ultracentrifuged (76,000 g, 4°C, 1 hour. MSE, England), diluted to the desired concentration and stabilized with BSA, 10mg/ml (Miles Laboratories, USA), prior to being aliquoted and stored at -20°C.

GAR-Ig : The same method as that of the RAM-$Ig_{(3)}$ iodination was used except that in some cases, the unbound iodine was removed by dialysis as follows: the labelled protein was transferred to a dialysis tube placed in a beaker containing 20ml 10% KI and 1 litre PBS, and left stirring at 4°C for 72 hours during which the beaker contents were twice replaced with PBS buffer.

Before use, 1ml amounts of all antisera were always preabsorbed with 20-40 x 10^6 CCH1 and/or T_3 Tc cells (4°C, 1-2 hours), ultra-

centrifuged (76,000 g, 1 hour, 4°C, MSE, England), and stored in aliquots at -20°C.

Cr^{51} LABELLING OF THE CELLS : To 5 x 10^5 cells/ml in Dulbecco saline 2.5mCi of ^{51}Cr was added (Amersham, England). The cellular suspension was incubated at 37°C for ½ hour shaking gently every 5 minutes. The cells were then washed three times with 20 ml 5% FCS-Dulbecco, the cells being recovered each time by centrifugation at 224 g, 4°C for 5 minutes, (MSE, England). The cells were left at 4°C for ½ hour to allow release of isotope from the cells, which were then again washed twice as above and resuspended into original volume in Dulbecco saline.

7. PREPARATION AND IODINATION OF IMMUNE RAM-Ig

Mouse Ig was prepared by precipitation with $(NH4)_2SO4$. After dialysis against coupling buffer, it was conjugated to CNBr activated sepharose (around 23 mg/g). 10 mls of RAM Ig(2) was applied to the sepharose column and unbound protein washed off with PBS. The specific antibody was acid eluted into the PBS buffer at PH 8.0. Approximately 6 mg of antibody was recovered. This was concentrated by vacuum dialysis to around 760 µgm/ml in saline and then aliquoted in 1 ml amounts.

1 ml of this reagent was iodinated with 1 mCi I^{125} using standard conditions. After purification on G-25 followed by ultracentrifugation in order to remove aggregates, the regant was diluted to 10 µgm/ml in complete RPMI containing 10 mg/ml BSA at PH 7.2.

NRS Ig was iodinated under identical conditions. It was observed that the antibody iodinated less readily than the normal IgG. The concentration

of each reagent was measured by single radial immunodiffusion after separation from free iodine by gel filtration. The concentration of each was found to be approximately the same.

8. THE INDIRECT PLATE RIAT

Freshly excised or cultured tumor cells (5 x 10^6/ml) were distributed in a plastic plate (Sterlin, Middlesex, England). 100/µl/well and centrifuged for 8 minutes at 224 g (MSE, England). 50 µl/well of RAM or NRS (1 µg/well) were added, left on ice for half an hour and the cells then washed three times with 10% FCS-MEM, being recovered after each washing by centrifugation at 224 g for 2 minutes at 4°C (MSE, England). 50 µl of ^{125}I-GAR (10 µg/well) was then added and the plate incubated at 4°C for another ½ hour. The cells were then washed three times with 5-10% FCS-PBS and transferred into a new plastic plate and left to dry overnight. The next day, the plate was cut into individual wells and counted. Individual tumors were tested in triple or quadruple wells.

9. THE INDIRECT TUBE RIAT

100 µl of freshly excised or cultured tumor cells (5 x 10^5/ml) were distributed in polyesterone tubes (Sterilin, U.K.) which had been precoated with 5% FCS by incubation overnight at 4°C, mixed with an equal volume of RAM (1 µg/100 µl) or NRS (3 µg/100 µl) and incubated on ice for one hour. The cells were then washed three times with 10% FCS MEM (4 ml/tub3) and incubated with 100 µl of ^{125}I-GAR (10 µg/100 µl) for a further one hour on ice. The cells were then washed four times with 5-10% FCS-PBS (4ml/tube) before being

counted. Individual tumors were studied in quadruple tubes.

10. THE DIRECT TUBE RIAT

100 µl of freshly excised or cultured tumor cells (5 x 10^5/ml) were distributed in 5% FCS precoated polyesterene tubes, mixed with an equal volume of ^{125}I-RAM (4 µg/100 µl) and incubated on ice for 1-2 hours. The cells were then washed four times with 5% FCS-PBS (4ml/tube), the cells being recovered after each wash by centrifugation at 224 g for 2-5 minutes at 4°C (MSE, England), and counted in Wallac gamma counter. Individual tumors were studied in quadruple tubes.

11. EVALUATION OF RESULTS

The GAR Ig bound to the cells in the indirect RIAT was determined in the following manner :

$$\text{amount of } ^{125}\text{I-GAR Ig bound} = \frac{\text{Counts/5 minutes/sample x 10,000 ng}}{\text{Counts/5 minutes of 100 µl of } ^{125}\text{I-GAR Ig}}^{*}$$

The RAM Ig bound to the cells in the direct method was similarly determined as follows :

$$\text{amount of } ^{125}\text{I-RAM Ig bound} = \frac{\text{Counts/5 minutes/sample x 4000 ng}}{\text{Counts/5 minutes of 100 µl of } ^{125}\text{I-RAM Ig}}^{*}$$

* The RAM Ig and GAR Ig were the TCA precipitates of the respective 100 µl radioiodinated reagents. The precipitates were obtained by adding 0.1 ml FCS and 4 mls 20% TCA to 0.1 ml of the iodinated reagent, and centrifuging the mixture at 1,133 g, 4°C, for 15 min. (MSE, England).

The antiglobulin bound represents the amount bound to freshly excised tumor cells less the amount bound to cultured tumor cells or empty tubes (controls).

R E S U L T S

THE DEVELOPMENT OF THE INDIRECT ANTIGLOBULIN TEST

The indirect radio-immune antiglobulin test (RIAT) extensively used in our department to study circulating antitumor antibodies was developed for the study of *in vivo* bound tumor associated immunoglobulins (TAIg). The development of the technique involved the following :

a) An assessment of the effect of enzyme treatments used in the preparation of tumor cell suspensions from freshly excised tumors. This was carried out in view of the ability of enzymes to remove surface bound TAIg.

b) A comparison between tube and plate methods in the indirect RIAT. The use of the tube method would have been preferred if it proved to be as sensitive as the plate method.

c) The effect of precoating the plates or the tubes with different sera on the inhibition of reagent binding to plastic.

d) The variation of reagent concentration, cell concentration and incubation time in order to determine the optimum conditions for TAIg studies.

e) Cell recovery studies in order to determine whether cell loss throughout the assay was correlated with the low radioactive counts obtained at the end of the RIAT.

f) An assessment of the influence of FCS in cell washings. This was carried out in view of the inhibition of non-specific binding attributed to FCS.

g) An investigation of nonspecific reagent binding to dead cells since low viability was a characteristic of the mechanically prepared cells.

h) A study of human aggregated IgG pretreatment of the cells to investigate reagent binding to infiltrating Fc-receptor host cells which may have been present in the excised tumor.

THE EFFECT OF ENZYME TREATMENT

Enzymes such as trypsin, pronase, and others are extensively used in the preparation of tumor cell suspension from freshly excised tumors. Certain enzymes are known to remove antigens and immunoglobulins from the surface of the cells (Hynes, 1976).

In order to determine whether enzyme treatment of the cells removed any TAIg that might be present on their surfaces, mechanically prepared cells were compared to those prepared by enzymes. In all the cases studied, enzyme prepared cells exhibited lower TAIg (Fig. 1).

In the first experiment, mechanically prepared CCH1 tumor cells were compared to cells prepared by pronase digestion. Mechanical preparation of the cells involved gentle teasing of tumor tissue and washing the cells in Dulbecco saline (see Materials and Methods). In contrast, enzyme preparation consisted of incubating the tumor pieces with the enzyme for 30 minutes at $37^{\circ}C$, then discarding the supernatant, followed by a further two or three 5 minute incubations where the supernatants were collected and the cells washed. As can be observed from Figure 1, pronase prepared cells exhibited a much lower TAIg level than mechanically prepared cells.

In the second experiment, in which mechanically prepared W54 "spontaneous" tumor cells were compared to those prepared by pronase, the latter once again showed lower TAIg levels. In both experiments 1 and 2, the enzyme concentration was 2.5 mg/ml.

In the third experiment, mechanically prepared CCH1 cells were compared to cells prepared by pronase and trypsin. As in the two previous experiments, enzyme prepared cells again showed lower levels of TAIg. The trypsin concentration was 2.5 mg/ml.

A COMPARISON OF PLATE AND TUBE METHODS

The plate method was compared to the tube method in order to determine which of the two methods was the more feasible for TAIg studies. The number of cells tested and the amounts of reagents used were the same in both methods. However, the volumes of the reagents used in the tube method were about twice as high. This facilitated cell washings and prevented excessive cell loss.

No significant difference in nonspecific binding was observed between the two methods (Fig. 2). However, in all experiments, the tube method consistently proved to be the more sensitive than the plate method.

A COMPARISON OF PRECOATING TECHNIQUES

High nonspecific binding of the reagents to the plastic was observed. In order to block or reduce this nonspecific binding, the plate was precoated with three different sera. The procedure consisted of precoating the wells of the plate with human, calf and horse sera (1 in 10 v/v) overnight at $4^{\circ}C$ and removing the sera before carrying out the RIAT.

Calf serum precoating was found to considerably reduce the nonspecific binding of the reagent to plastic (Fig. 3). In contrast, however, the

reagents were found to cross react with the horse and human sera, thus leading to an increase of the nonspecific binding.

In a second experiment, precoating with 5% foetal calf serum (FCS) was found to substantially reduce the nonspecific binding of the reagent to plastic (Fig. 8).

Since precoating with FCS reduced nonspecific binding, it was adopted as a standard procedure for the application of RIAT in TAIg studies.

AN EXAMINATION OF CELL RECOVERY

The ^{125}I GAR counts obtained were relatively low. One reason behind this could have been the loss of cells throughout the RIAT. In order to examine this probability, Cr_{51} labelling was used.

The Cr_{51} labelling procedure has been mentioned in the materials and methods section. Briefly, 5 x 10^5 cells/ml were labelled with 2-3 mCi Cr_{51} sodium chromate for 30 minutes at 37°C, washed 3 times, left at 4°C for ½ hour and then washed again 2 times. The RIAT was then carried out as mentioned earlier. The process consisted of incubating the cells with RAM and NRS for ½ hour on ice, washing the cells three times, then incubating the cells with ^{125}I-GAR for a further ½ an hour. The cells were then transferred to a new plate after washing them 3 times. Untransferred cells were also studied.

In the first experiment, CCH1 20 tumor cells were studied and a general reduction of Cr_{51} counts was observed throughout the RIAT. The trend obtained was as follows in decreasing order : untreated cells, cells washed 3 times, cells incubated twice and washed 6 times

(untransferred), cells incubated twice and washed 6 times (transferred to new wells). As can be observed, similar counts were obtained in the presence of the three reagents NRS, RAM, GAR (Fig. 4).

In the second experiment, cultured cells were studied. Since the cells had a higher viability than the mechanically prepared CCH1 cells, they produced relatively higher counts. The general trend, however, remained very similar to that of the previous experiment (Fig. 5).

Although these results might suggest loss of cells throughout the RIAT, it is more likely due to spontaneous release of Cr_{51}.

THE VARIATION OF REAGENT CONCENTRATION

Six experiments were carried out to study the variations of reagent concentration. In all cases a positive correlation of reagent concentration with its binding to the cells was obtained.

The concentration of ^{125}I GAR Ig, was studied in the first experiment where dilutions of 25, 50, 75 and 100 µg/ml concentrations were used. Almost a direct relationship of reagent concentration to its binding to the cells was obtained between 25 and 75 µg/ml. However, the slope of the variation became less acute as the concentration was increased to 100 µg/ml (Fig. 6).

In the second experiment, binding of the GAR Ig to the cells was once again found to vary directly with its concentration in both the plate and tube methods (Fig. 7). Unlike the previous experiment, however, the slope of the variation remained steep even though high concentrations of the reagent were used.

In the remaining four experiments, the variation of RAM Ig and NRS concentrations were assessed. As can be observed from Figure 8,

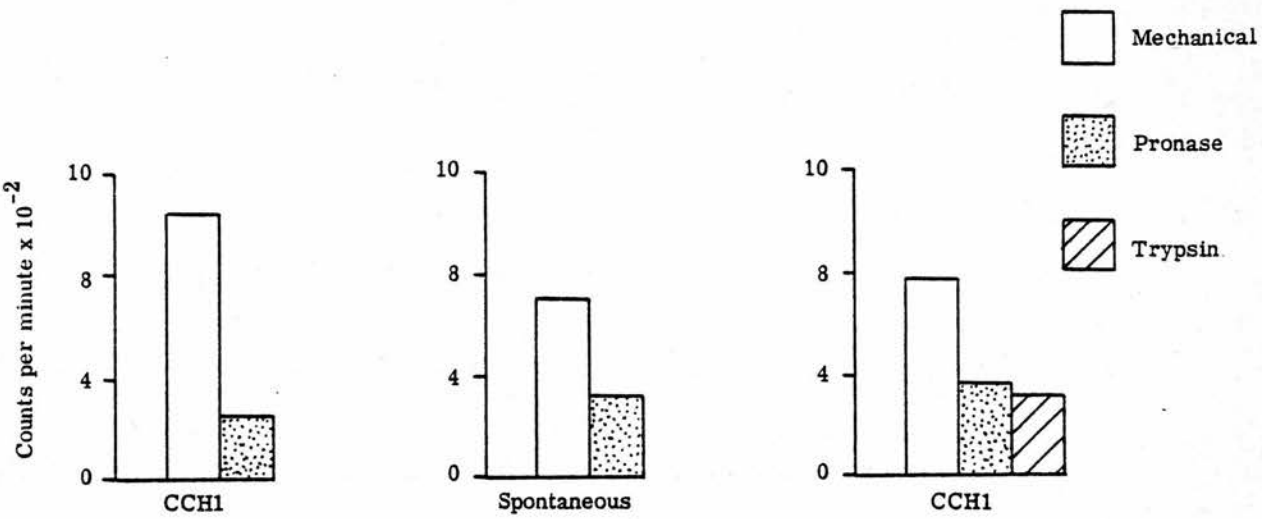
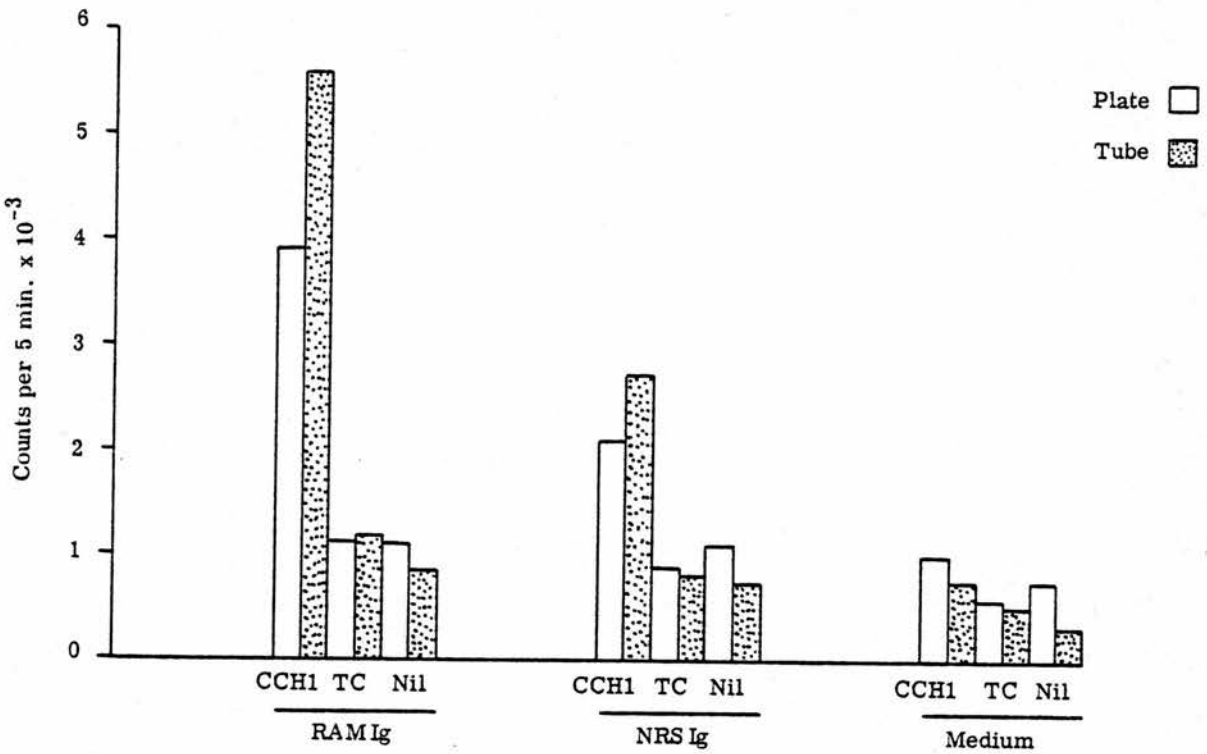


FIG 1 A COMPARISON BETWEEN MECHANICAL AND ENZYME PREPARATIONS OF TUMOR CELLS

Mechanical preparation consisted of teasing the tumor tissue with a sterile syringe needle at 0-4°C. Enzyme preparation included the routine enzyme procedure outlined in the 'Materials and Methods' section. The figure depicts the results of 3 experiments carried out on freshly excised CCH1 and W54 'spontaneous' tumor cells by the indirect plate radioimmune antiglobulin test. Note that enzyme treated tumor cells had lower TAIg levels than mechanically prepared tumor cells.



IG 2 A COMPARISON BETWEEN THE PLATE AND TUBE INDIRECT RADIO-IMMUNE ANTIGLOBULIN TESTS.

Equal amounts of reagents and number of tumor cells were used in both the plate and tube methods. However, the volume of the medium in which the cells and reagents were suspended in the tube method were about 3 times more than that of the plate method. Moreover, the tube method enabled the use of larger volumes of washing media. It can be observed that the specificity of RAM Ig binding towards freshly excised tumor cells (CCH^1) is increased in the tube method. Note that reagent binding to cultured tumor cells (TC) hardly exceeds background (Nil) levels. NRS Ig binding to freshly excised cells may be due to infiltrating host cells.

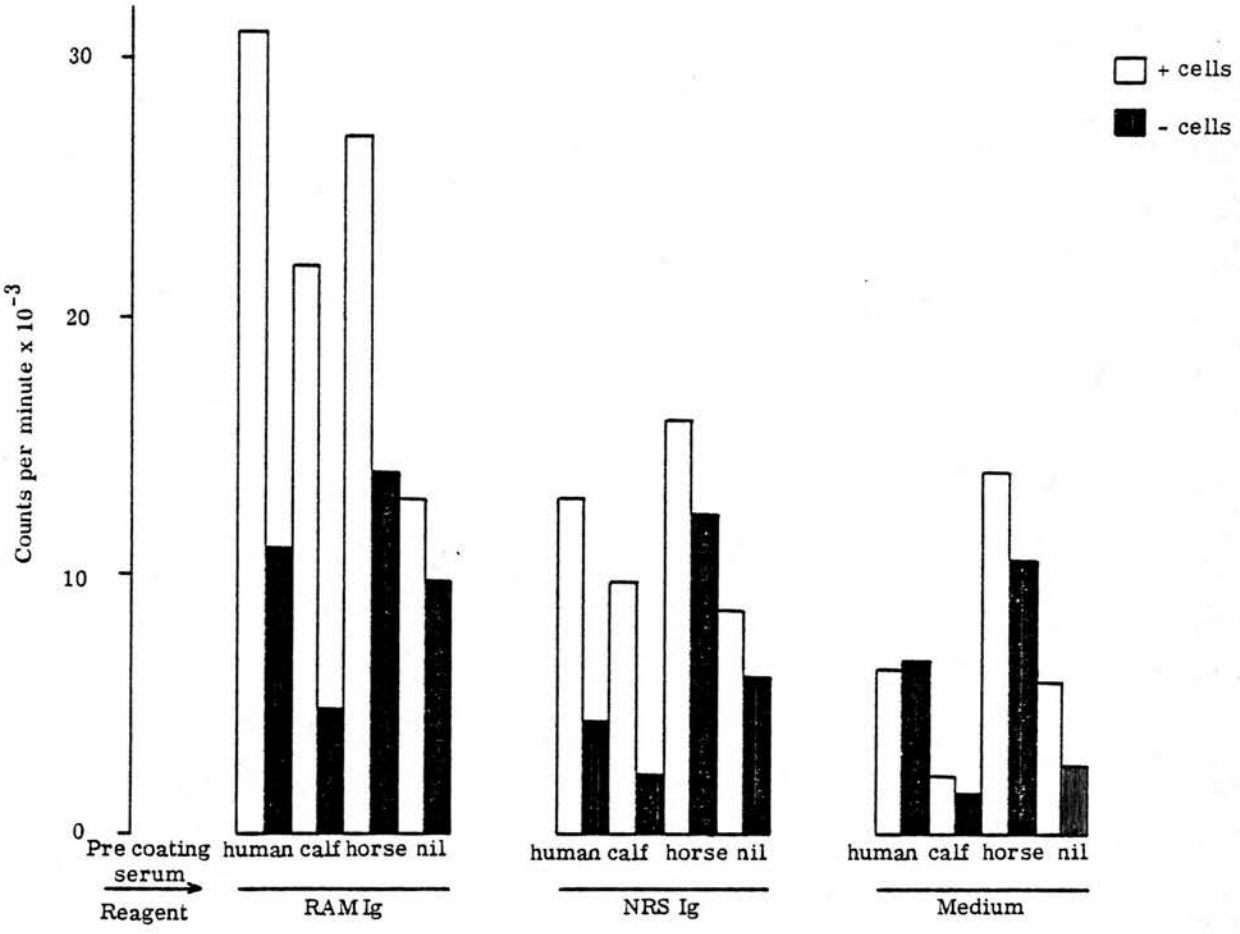


FIG 3 THE EFFECT OF PRECOATING PLATES WITH VARIOUS PROTEINS ON THE NONSPECIFIC BINDING OF REAGENTS

The wells in the plastic plate were precoated with human, calf and horse sera overnight at $4^{\circ}C$. The sera were then decanted and the standard indirect radio-immune antiglobulin test carried out on freshly excised CCH1 tumor cells and controls (-cells). It can be observed that human and horse sera enhanced the nonspecific binding of RAM Ig in contrast to calf serum which inhibited this nonspecific binding (compare precoated nil wells with nonprecoated nil wells). Similarly, nonspecific binding of NRS Ig and ^{125}I-GAR Ig (medium) was most inhibited by calf serum. NRS Ig binding to tumor cells may be due to infiltrating host cells.

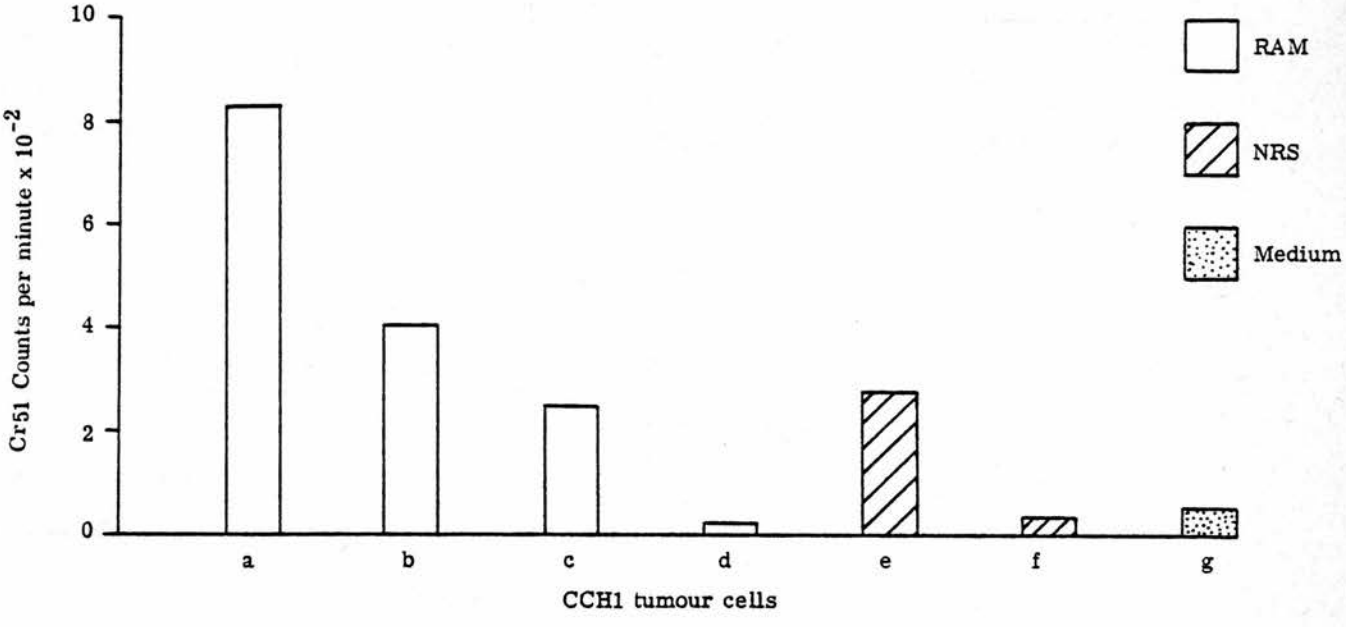


FIG 4 AN EXAMINATION OF CELL RECOVERY

Freshly excised CCH1 tumor cells were prelabelled with ^{51}Cr and their progress through the standard indirect plate radio-immune antiglobulin test was assessed. It can be observed that the counts decrease as the assay progresses: a. untreated cells; b. cells after the first incubation and 3 washes; c. cells after the second incubation and 6 washes; d. cells after the final transfer to new wells. Note that similar counts were observed with cells incubated with NRS Ig or medium; e. cells after the second incubation and 6 washes; f & g. cells after the final transfer to new wells.

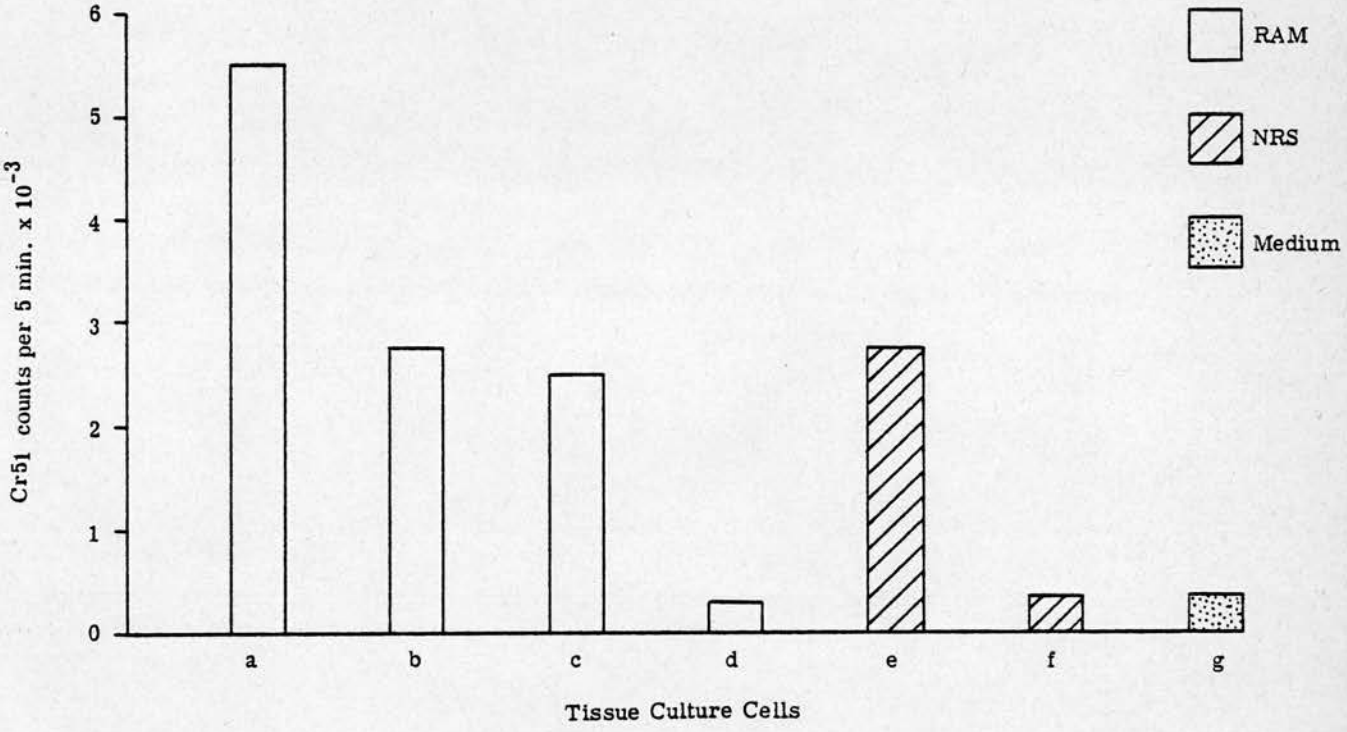


FIG 5 AN EXAMINATION OF CELL RECOVERY

Cultured CCH1 tumor cells were prelabelled with ^{51}Cr and their progress through the standard indirect plate radio-immune antiglobulin test was assessed. It can be observed that the counts decrease as the assay progresses: a. untreated cells; b. cells after the first incubation and 3 washes; c. cells after the second incubation and 6 washes; d. cells after the final transfer into new wells. Note that similar counts were observed with cells incubated with NRS Ig or medium; e. cells after the second incubation and six washes; f & g cells after the final transfer to new wells.

the results of the first experiment exhibited an optimum value of 1 in 50, although the probability of an artifact could not be excluded.

In the second experiment, where RAM and NRS dilutions of 1, 0.5 and 0.25 µg/100 µl concentrations were studied, a direct correlation of reagent binding to cells with reagent concentration was observed in both the plate and tube methods. Although higher binding was obtained with the tube method, the general trend of the variation was similar to that observed with the plate method (Fig. 9).

In the third experiment, RAM and NRS dilutions of 5 and 1 µg/100 µl concentrations were studied. Once again a direct relationship of binding with reagent concentration was noted (Fig. 10).

Finally, in the fourth experiment, RAM and NRS amounts of 100 and 10 µg were studied. As can be observed from Figure 11, reagent binding to cells varied positively with its concentration.

THE VARIATION OF CELL NUMBER

The standard number of cells used in our assay was 5×10^5/ml. However, in order to determine whether reagent binding was dependent on cell number, the amount of binding to two different cell numbers of 5×10^6/ml and 5×10^5/ml was studied. The binding of the reagent to the cells was found to be directly proportional to the cell number (Fig. 11).

THE VARIATION OF INCUBATION TIME

The indirect RIAT involved two incubation periods (See Material and Methods). In the first incubation period RAM and NRS reacted with the cells and in the second incubation period the GAR Ig reacted with the

cells. In both cases the reagent binding was found to increase as the incubation time was prolonged.

The incubation time of RAM and NRS with the cells was studied in the first experiment where incubation periods of 15, 30, 60 and 120 minutes were used. In both the plate and tube methods, reagent binding to the cells was found to increase as the incubation time was prolonged (Fig. 12). The drop observed at 60 minutes with the plate method could have been an artifact.

In the second experiment the incubation time variation of GAR Ig with the cells was studied. Again binding increased as the incubation time was prolonged (Fig. 13).

THE EFFECT OF HUMAN AGGREGATE IgG PRETREATMENT

Fc-receptor host cells have been found to be present within tumors and tumor cell suspensions (Szymaniec and James, 1976). In order to find out whether our reagents were binding to the Fc-receptors of host cells, two experiments were carried out in which excised and cultured CCH1 cells were pretreated with aggregated human IgG for ½ hour at 4°C. This pretreatment was expected to block the Fc-receptors of the cells and thus prevent the Fc-portion of the RAM and/or GAR Ig from binding to these receptors. The RAM and NRS had been absorbed with the aggregated IgG.

In the two experiments that were carried out to examine reagent binding to Fc-receptors, no significant difference between aggregated IgG pretreated and nontreated cells was observed (Figs. 10 and 14).

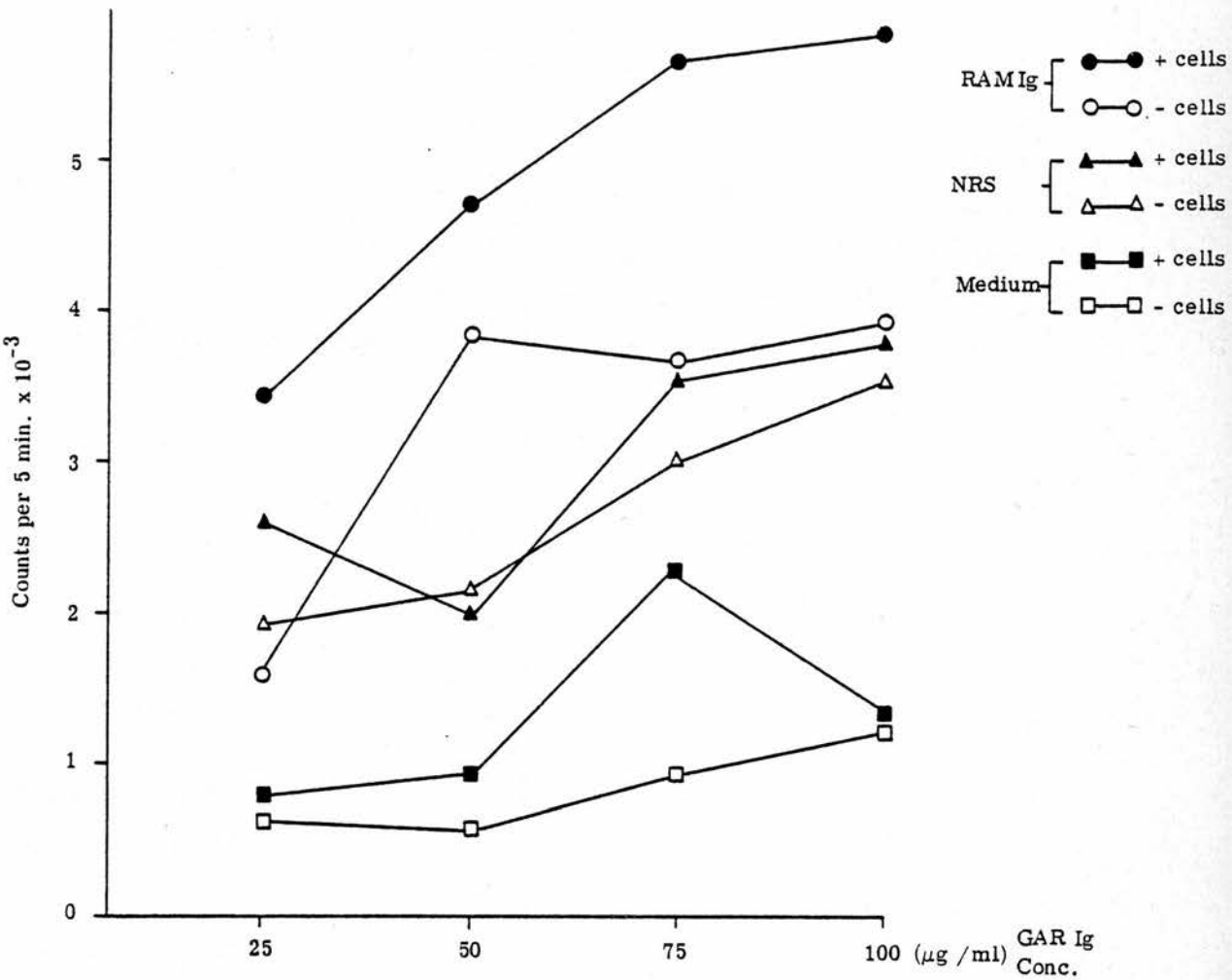


FIG 6 DETERMINATION OF THE OPTIMUM CONCENTRATION OF ^{125}I-GAR Ig IN THE INDIRECT PLATE RADIO-IMMUNE ANTIGLOBULIN TEST

In this experiment, the concentration of GAR Ig was varied while RAM Ig and NRS Ig concentrations were kept constant. Note that the binding of ^{125}I-GAR Ig to the freshly excised CCH1 tumor cells incubated with RAM Ig, NRS Ig, or medium, usually increases with the concentration of the labelled reagent until it reaches a plateau.

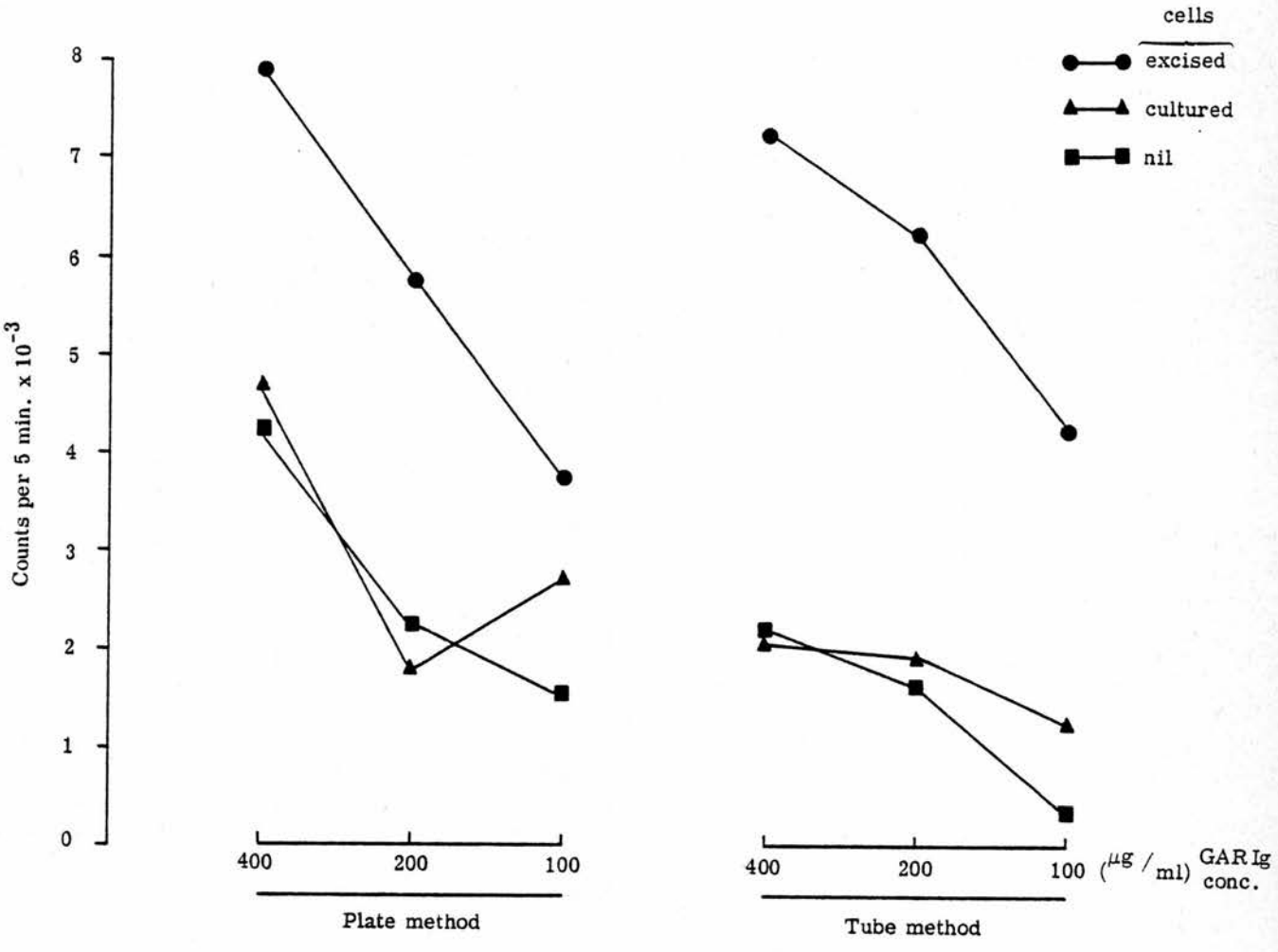


FIG. 7 DETERMINATION OF THE OPTIMUM CONCENTRATION OF ^{125}I-GAR Ig IN THE INDIRECT PLATE AND TUBE RADIO-IMMUNE ANTIGLOBULIN TESTS.

In this experiment, the concentration of GAR Ig was varied while the RAM Ig concentration was kept constant. It can be observed that the binding of ^{125}I-GAR Ig to freshly excised tumor cells increases with its concentration. Note that reagent binding to cultured tumor cells is hardly above background levels.

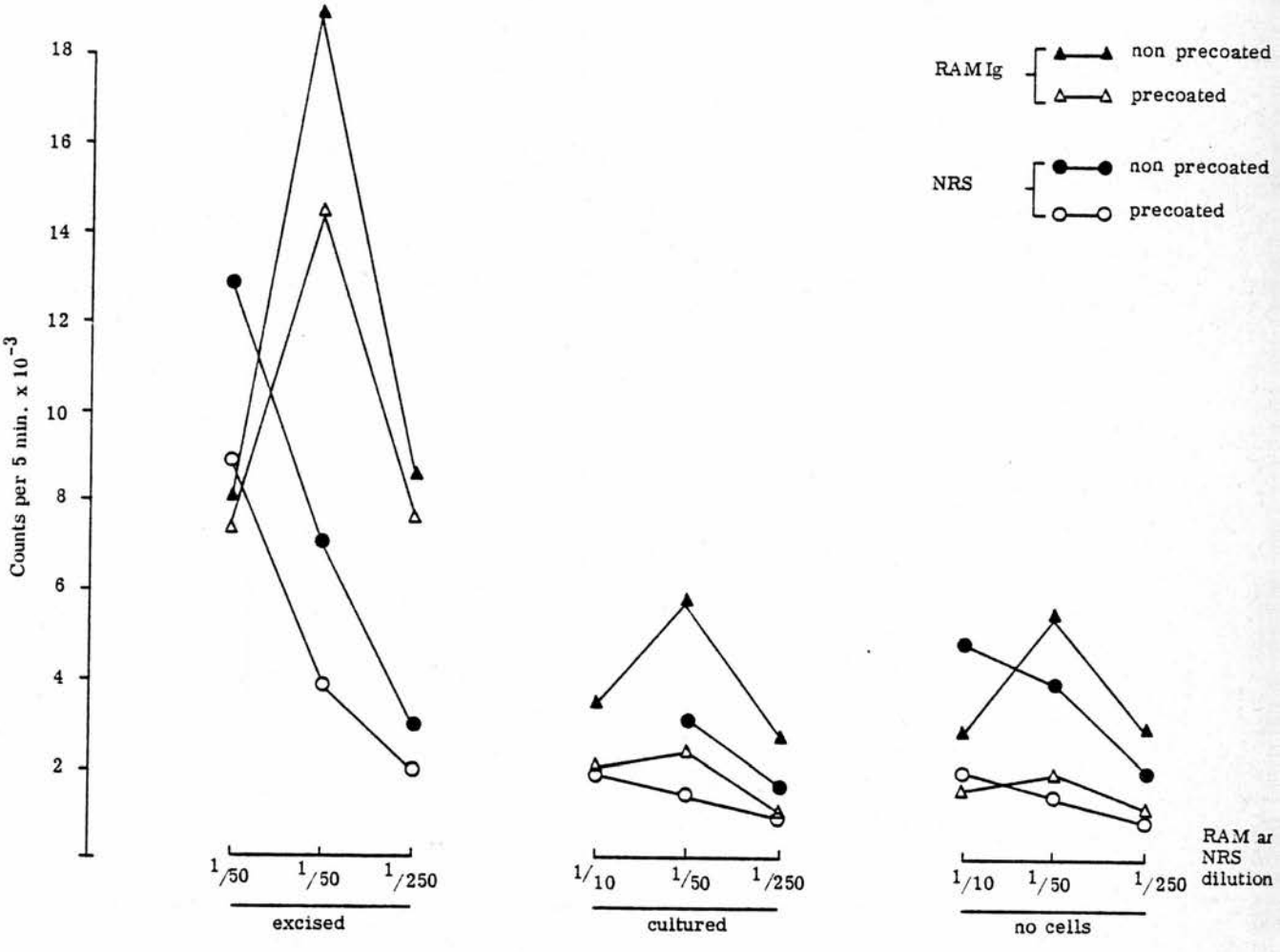


FIG 8 DETERMINATION OF THE OPTIMUM DILUTIONS OF RAM Ig and NRS Ig IN THE INDIRECT TUBE RADIO-IMMUNE ANTIGLOBULIN TEST

In this experiment, the dilutions of RAM Ig and NRS Ig were varied while the GAR Ig concentration was kept constant. Both nonprecoated tubes and tubes precoated with 5%-FCS-PBS were used. In this experiment, the optimum RAM Ig dilution was found to be 1:50. Note that precoating inhibited the nonspecific binding of reagents to plastic (no cells). Also note that reagent binding to cultured tumor cells was hardly above background levels.

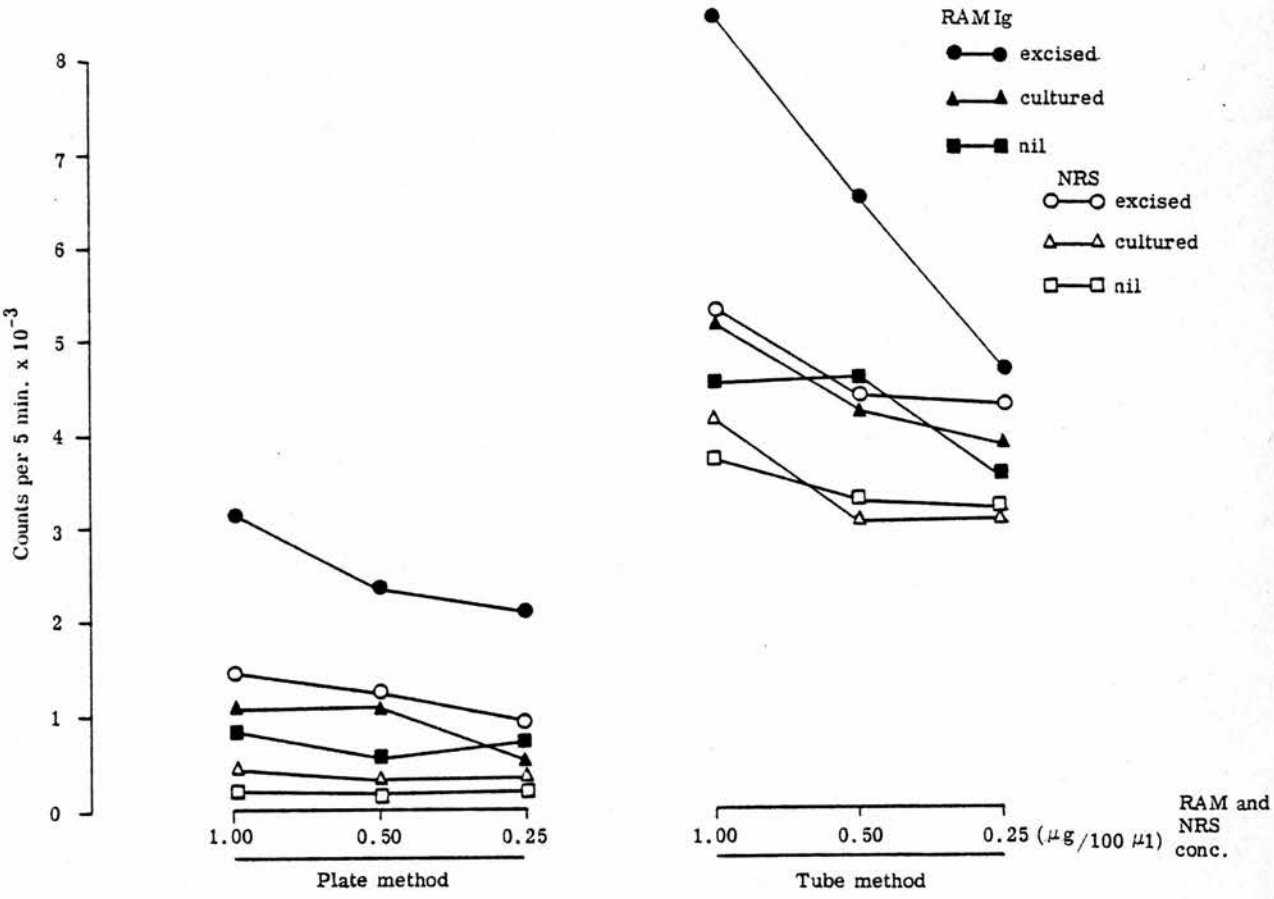


FIG 9 DETERMINATION OF THE OPTIMUM AMOUNTS OF RAM Ig and NRS Ig IN THE INDIRECT PLATE AND TUBE RADIO IMMUNE ANTIGLOBULIN TESTS

In this experiment, the amounts of RAM Ig and NRS Ig were varied while the GAR Ig concentration was kept constant. In both the plate and tube methods GAR Ig binding to the freshly excised cells increases with the amount of RAM Ig. Note that reagent binding to cultured tumor cells does not exceed background (nil) levels.

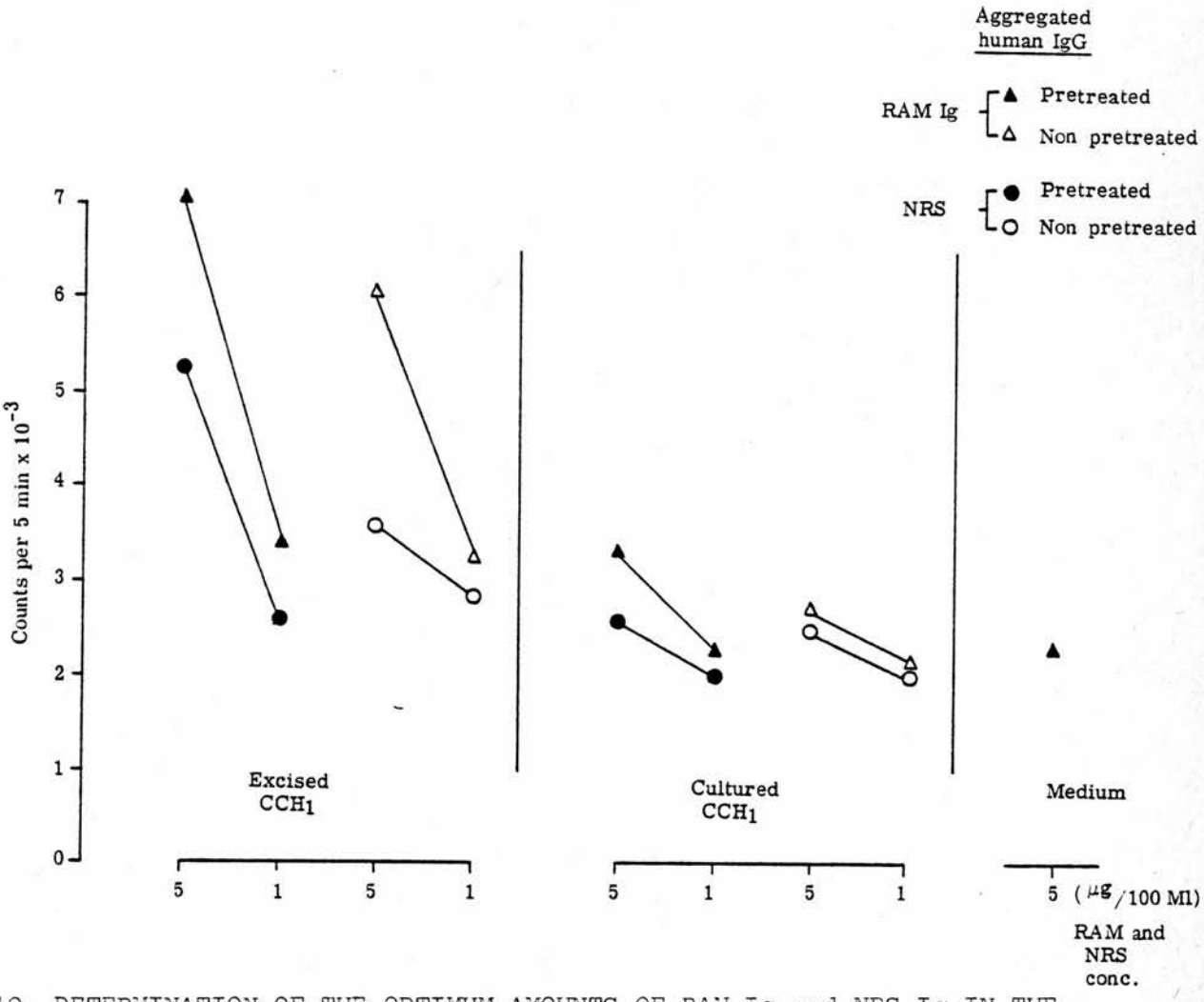


FIG 10 DETERMINATION OF THE OPTIMUM AMOUNTS OF RAM Ig and NRS Ig IN THE INDIRECT TUBE RADIO IMMUNE ANTIGLOBULIN TEST, AND THE EFFECT OF TREATMENT WITH AGGREGATED HUMAN IgG

In this experiment, the amounts of RAM Ig and NRS Ig were varied while the GAR Ig amount was kept constant. Moreover, the freshly excised and cultured CCH1 tumor cells were incubated with either medium or aggregated human IgG before being tested in the radio immune antiglobulin test. It can be observed that GAR Ig binding to cells increases with the amounts of RAM Ig and NRS Ig. Note that pretreatment with aggregated human IgG did not decrease reagent binding to the cells. This suggests that the reagent binding is not due to cytophyllic Fc-interactions. It does not, however, exclude the possibility that part of the interaction is due to nonspecific antibodies

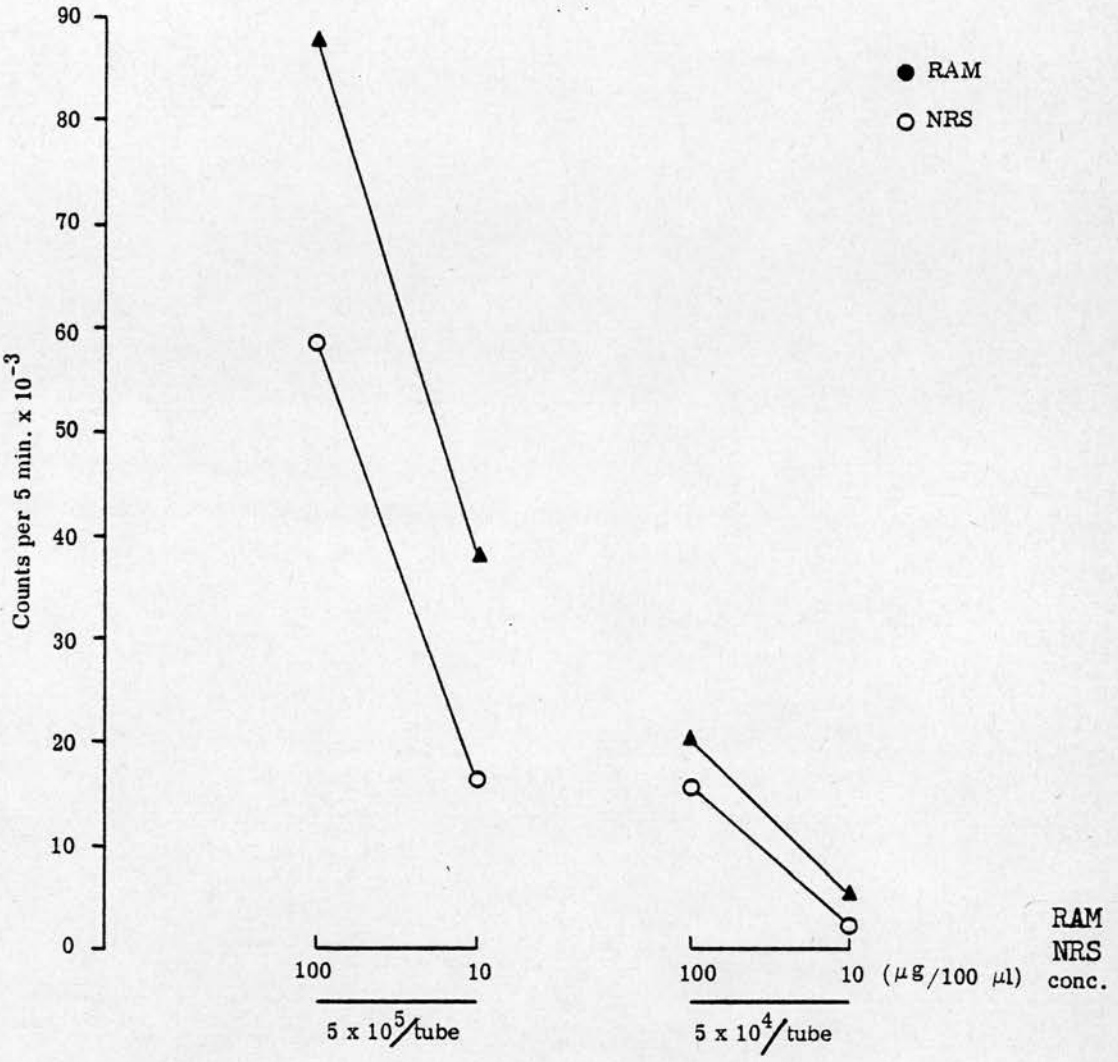


FIG 11 DETERMINATION OF THE OPTIMUM AMOUNTS OF RAM Ig and NRS Ig IN THE INDIRECT TUBE RADIO IMMUNE ANTIGLOBULIN TEST AT TWO DIFFERENT CELL NUMBERS

In this experiment, the amounts of RAM Ig and NRS Ig were varied while the GAR Ig amount was kept constant. Moreover, the effect of cell number was assessed. It can be observed that GAR Ig binding to cells increases with the amounts of RAM Ig and NRS Ig. Note that reagent binding also varies positively with the number of the freshly excised tumor cells.

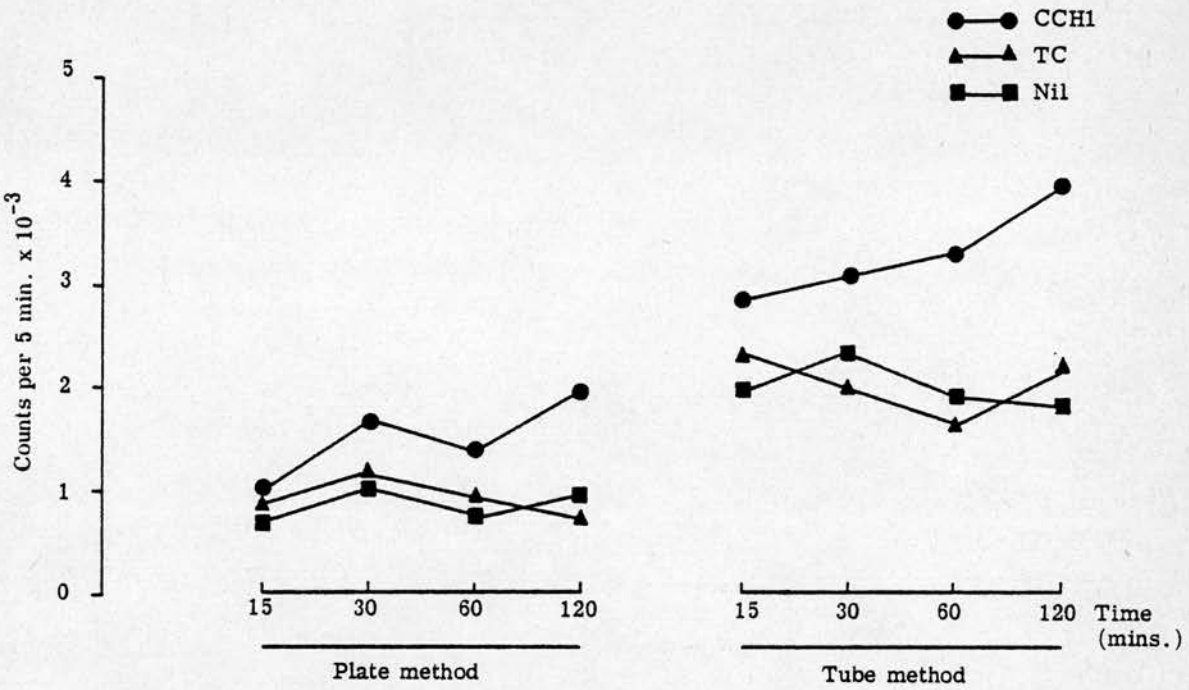


FIG 12 DETERMINATION OF THE OPTIMUM FIRST INCUBATION TIME IN THE INDIRECT PLATE AND TUBE RADIO IMMUNE ANTIGLOBULIN TESTS

In this experiment, the reagent concentrations were kept constant while the time of the first incubation period was varied. In both the plate and tube methods, reagent binding to freshly excised CCH1 tumor cells gradually increased with incubation time. Note that reagent binding to TC (cultured CCH1 cells) does not exceed background levels.

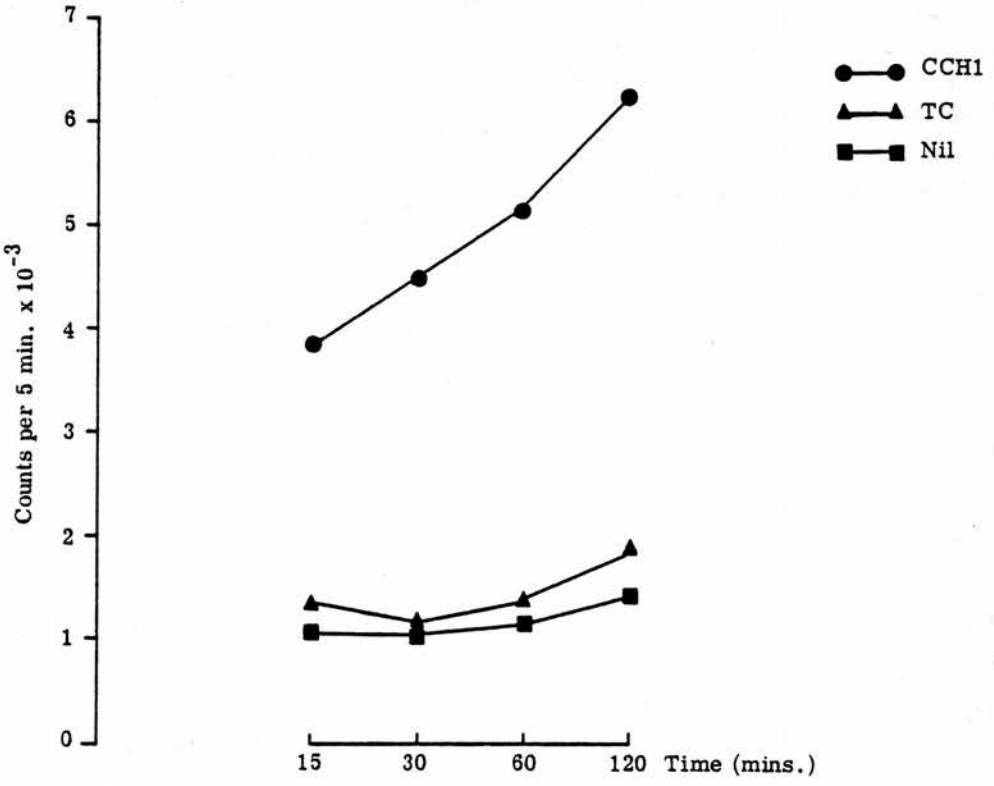


FIG 13 DETERMINATION OF THE OPTIMUM SECOND INCUBATION TIME IN THE INDIRECT TUBE RADIO IMMUNE ANTIGLOBULIN TEST

In this experiment, the reagent concentrations were kept constant while the time of the second incubation period was varied. It can be observed that the reagent binding to freshly excised CCH1 tumor cells gradually increased with incubation time. Note that reagent binding to TC (cultured CCH1 cells) does not exceed background levels.

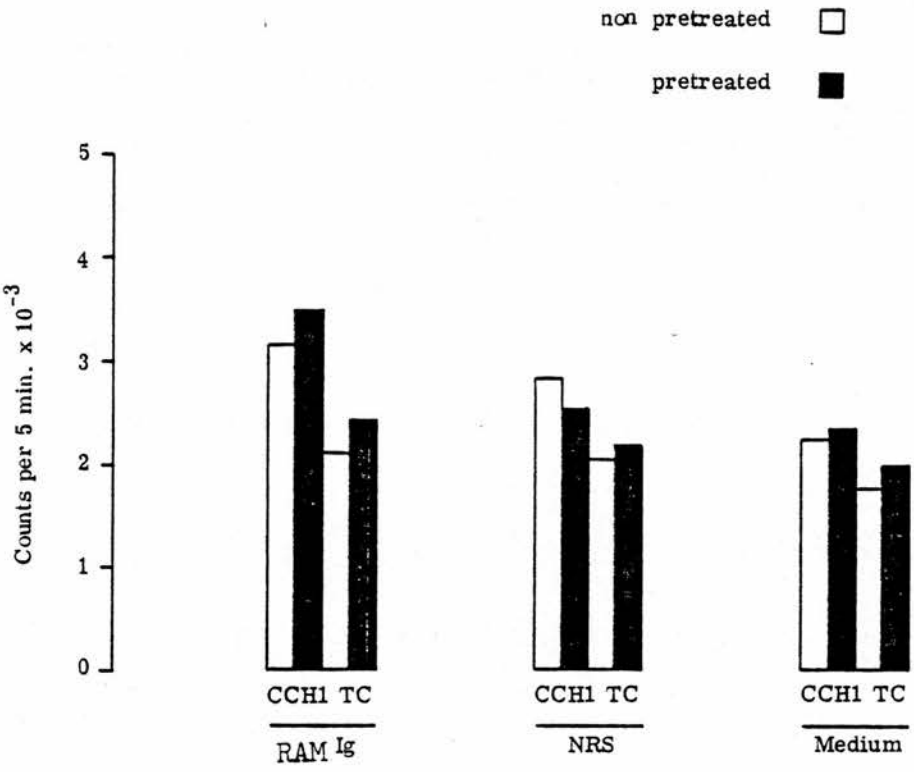


FIG 14 THE EFFECT OF TREATMENT WITH AGGREGATED HUMAN IgG ON REAGENT BINDING IN THE INDIRECT PLATE RADIO IMMUNE ANTIGLOBULIN TEST

Freshly excised and cultured CCH1 tumor cells were pretreated with aggregated human IgG for half an hour at $4^{\circ}C$, separated from the aggregated IgG by centrifugation, and then compared to nontreated cells in the indirect plate radio immune antiglobulin test. Note that no significant difference was observed between treated and non-treated cells.

NONSPECIFIC BINDING TO DEAD CELLS

Since the mechanically prepared cells had low viability, the possibility of nonspecific binding to dead cells was investigated. Binding to both killed excised and cultured tumor cells was studied.

Mechanically prepared cells were divided into two groups, one of which was stored at $4^{\circ}C$ for 2 hours while the other group was incubated at $48^{\circ}C$ for 2 hours. At the end of the 2 hours, the viability of the first group remained 40% whereas the viability of the second group was reduced to 15%. As can be observed from Figure 15, no significant difference of binding was obtained between the two groups.

In the second experiment, dead and live tissue culture cells (TC) were compared. The live cells were killed by a two hour incubation period at $48^{\circ}C$ which reduced their viability from 100% to 12%. No significant difference of reagent binding between live and dead TC was obtained (Fig. 16).

THE EFFECT OF FCS IN CELL WASHINGS

Since FCS was found to inhibit nonspecific binding to plastic, its role in cell washings was examined. As previously mentioned, the indirect RIAT involved two groups of washings: one group after the first incubation period which consisted of three washings with 10% FCS-MEM (4ml/tube), and another group after the second incubation period which consisted of four washings with 10% FCS-PBS (4ml/tube).

A comparison of 5% FCS medium with 10% FCS medium was carried out in the first experiment. The medium which contained 10% FCS was found to produce less nonspecific binding than the medium which contained 5% FCS (Fig. 17).

Media containing 10% FCS, 5% FCS and no FCS were compared in the second group of washings. No significant difference in nonspecific binding was obtained between media containing 10% FCS or 5% FCS (Fig. 18).

Based on these results, it was therefore agreed to use media containing 10% FCS in the first group of washes, and media containing 5% FCS in the second group of washes.

SUMMARY

Studies were undertaken to determine the optimal experimental conditions for measuring tumor associated Ig by the indirect RIAT.

Preliminary studies revealed as expected the unfeasibility of using enzymes for tumor cell preparation as they were found to remove the surface bound TAIg. Consequently, the enzyme procedure was replaced by mechanical preparation of the cells.

A comparison of plate and tube methods showed the tube method to be a more sensitive and feasible method for the study of TAIg.

Precoating the tubes with 5% FCS was found to appreciably inhibit the nonspecific binding of reagents to the plastic.

Reagent binding to cells was found to be directly correlated with reagent concentration, cell number and incubation time. The following conditions were found to give satisfactory results :

a) GAR Ig concentration of 10 μg/100 μl

b) RAM Ig concentration of 1 μg/100 μl

c) cell number of 5×10^4/100 μl

d) incubation time of 1 hour

Cell recovery studies appeared to suggest spontaneous ^{51}Cr release and/or cell death throughout the RIAT.

A study of cell washing media showed 10% FCS-MEM and 5% FCS-PBS to be the most effective media.

Dead cells were found not to bind the reagents nonspecifically. The high binding to mechanically prepared cells could not therefore be attributed to nonspecific binding to dead cells.

Finally, reagent binding to Fc-receptor host cells within the tumor was found to be insignificant.

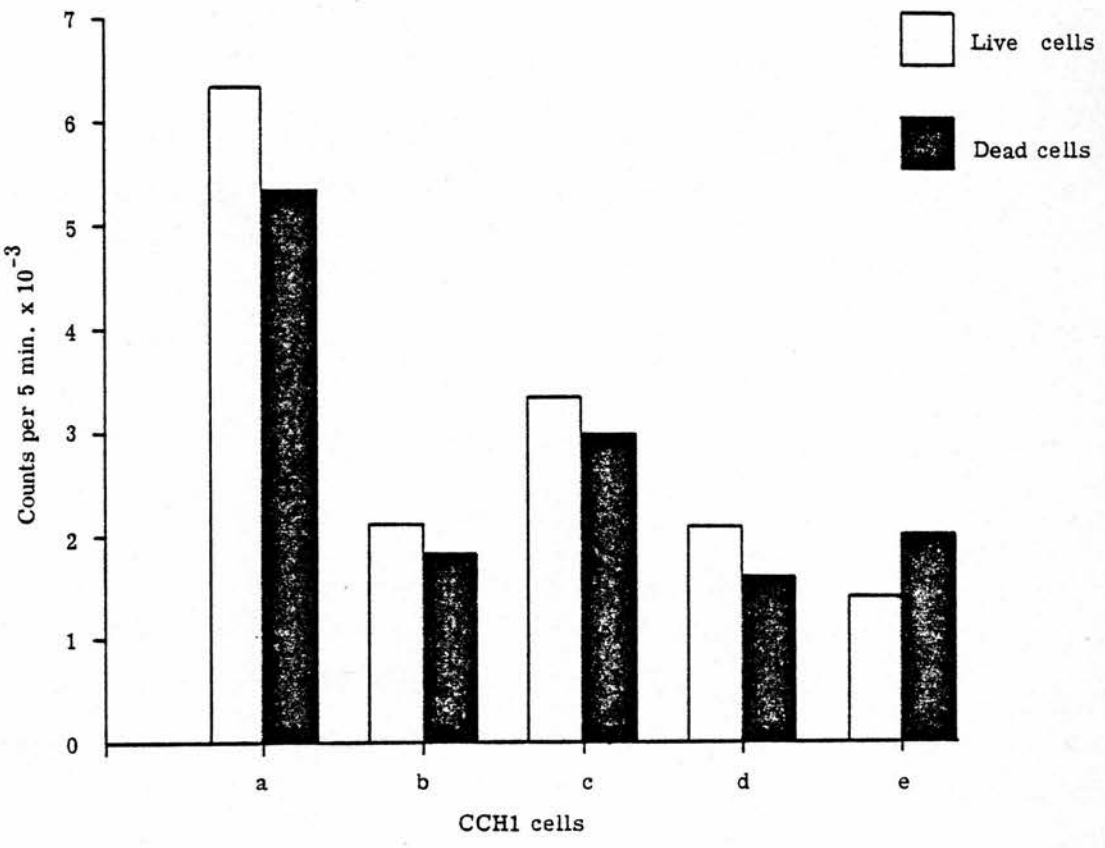


FIG 15 THE EFFECT OF DEAD CELLS ON REAGENT BINDING

Freshly excised CCH1 tumor cells were divided into 2 tubes, one of which was left at 4°C for 2 hrs, while the other was incubated in a 48°C water bath for 2 hrs. The viability of the cells incubated at 4°C was 40% and they were denoted as 'live cells'. The viability of the cells incubated at 48°C was 15% and they were denoted as 'dead cells'. The indirect plate radio immune antiglobulin test was carried out on the 'dead'and 'live' cells. It can be observed that at the various final stages of the radio immune antiglobulin test, there was no increased binding of reagents to 'dead' cells: (a) and (b); cells incubated with RAM Ig before and after transfer into new wells respectively. (c) and (d); cells incubated with NRS Ig before and after transfer into new wells respectively (e); cells incubated with medium after the transfer into new wells.

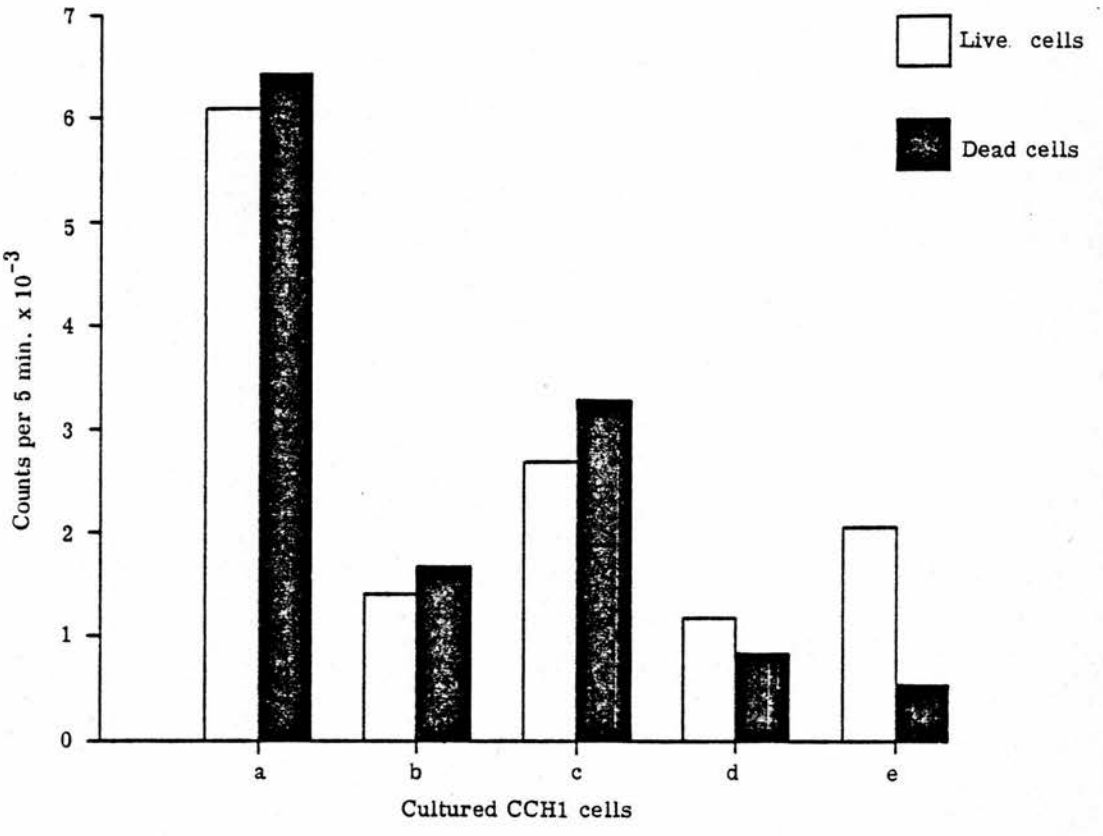


FIG 16 THE EFFECT OF DEAD CELLS ON REAGENT BINDING

Cultured CCH1 tumor cells were divided into 2 tubes, one of which was left at 4°C for 2 hrs, while the other was incubated in a 48°C water bath for 2 hrs. The viability of the cells incubated at 48°C dropped from 100% to 12%. The indirect plate ratio-immune antiglobulin test was carried out on the live and dead cells. It can be observed that at the various final stages of the radio immune antiglobulin test, there was no increased binding of reagents to dead cells: (a) and (b); cells incubated with RAM Ig before and after transfer into new wells respectively (c) and (d); cells incubated with NRS Ig before and after transfer into new wells respectively (e); cells incubated with medium after transfer into new wells.

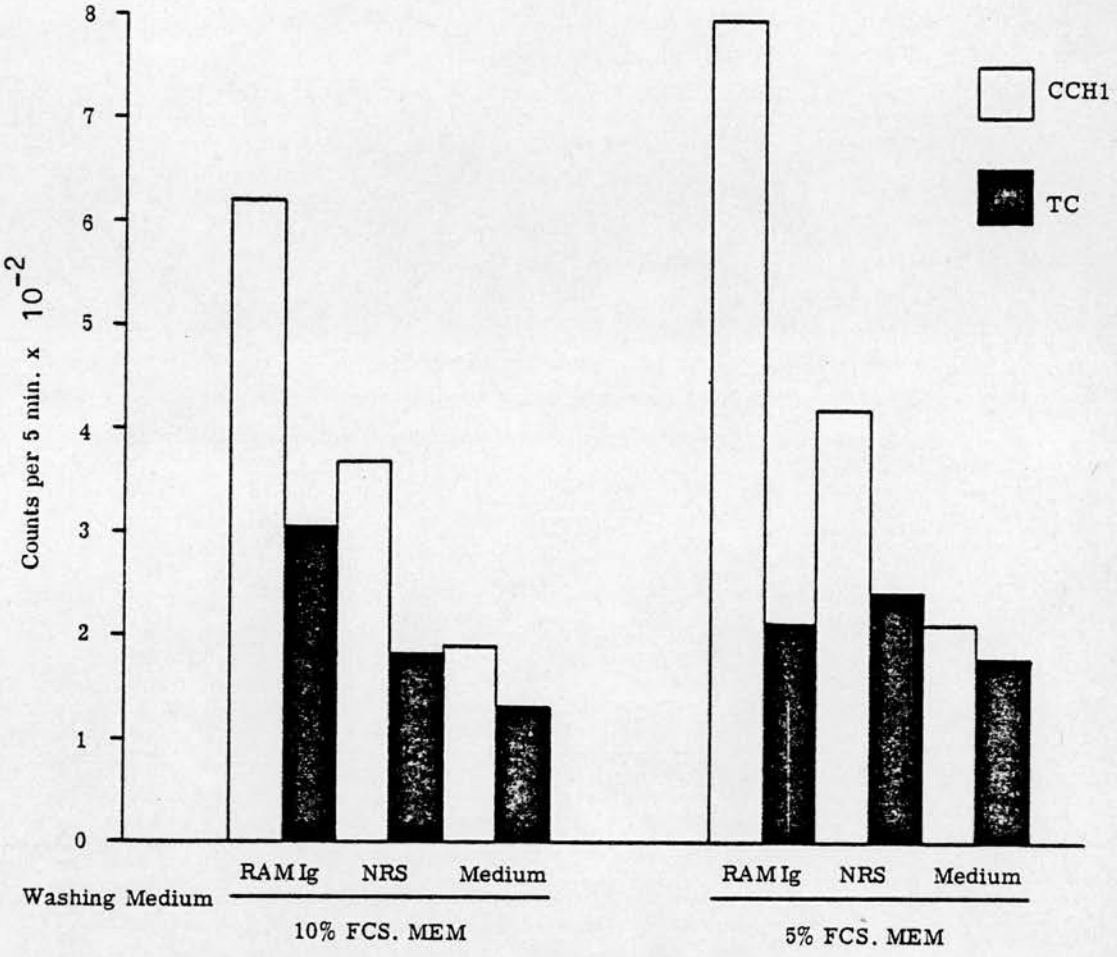


FIG 17 THE EFFECT OF FCS IN WASHING MEDIA IN THE INDIRECT TUBE RADIO IMMUNE ANTIGLOBULIN TEST

In this experiment, all conditions were kept constant while the FCS content in the washing media used after the first incubation period was varied. Although no significant differences were noted between the use of the different media, it can be observed that washing with 5% FCS MEM slightly reduced nonspecific binding of RAM Ig to control tubes (TC)

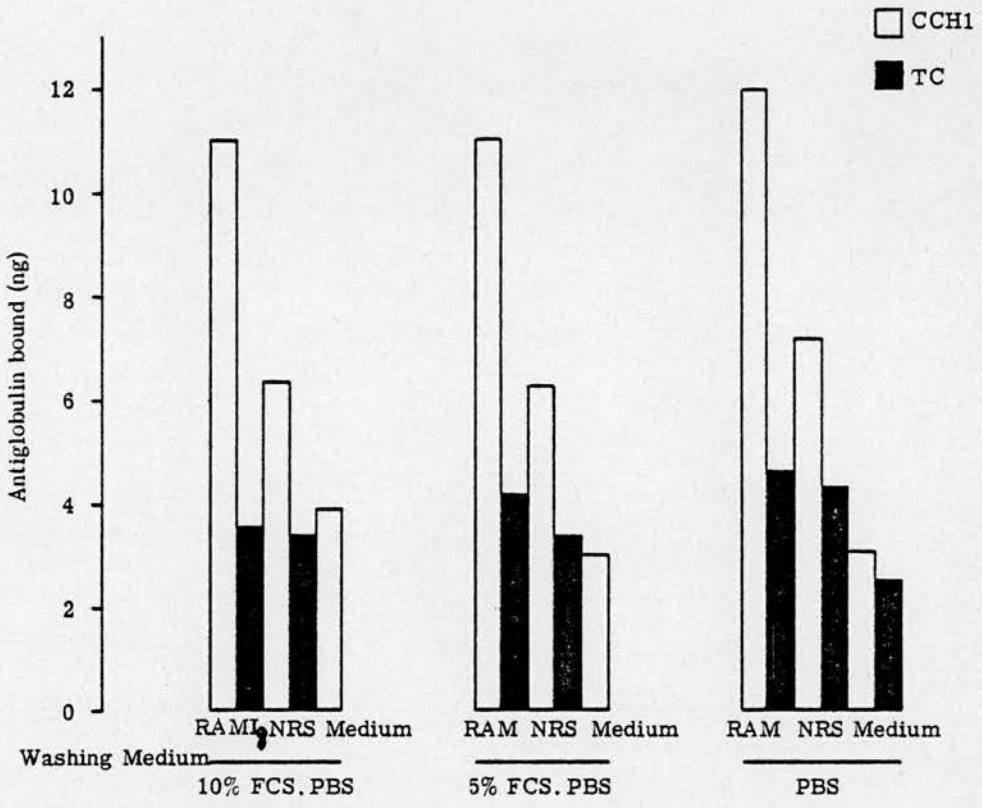


FIG 18 THE EFFECT OF FCS IN WASHING MEDIA IN THE INDIRECT TUBE RADIO IMMUNE ANTIGLOBULIN TEST

In this experiment, all concentrations were kept constant while the FCS content in the washing media used after the second incubation period was varied. It can be observed that washing with FCS containing media slightly reduced the nonspecific binding of reagents to control tubes (TC)

THE APPLICATION OF THE INDIRECT ANTIGLOBULIN TECHNIQUE

THE RESPONSE TO CCH 1 IN NONTREATED AND C. PARVUM TREATED MICE

Four experiments were carried out in order to study the variation of *in vivo* bound tumor associated Ig (TAIg) to cells with time and size of tumors in nontreated and *C. parvum* (cp) treated mice. The treated mice included those which had been injected with 0.2 ml (1.4 mg/ml) cp (Wellcome Research Laboratories, England) intraperitoneally 3 days after the injection of tumor cells.

In the first experiment, tumor cells from nontreated mice showed a slight increase of TAIg binding on the third week, while those of cp treated mice showed a substantial increase on week three, followed by a decrease on week four. However, the standard deviation (SD) remained high in both groups.

The results of the second experiment showed a different pattern. The tumors from nontreated mice exhibited a slight decrease of TAIg on week 3, while those of cp treated mice showed a continual increase with time. Nevertheless, the SD in both groups still remained high.

In experiment 3, tumors from nontreated mice showed a different pattern from cp treated mice. Whereas in the former the TAIg increased on week 3 and decreased on week 4, in the latter the TAIg decreased and increased on weeks 3 and 4 respectively (Fig. 19).

The results of the last experiment showed an increase of TAIg with time in both groups (Fig. 20). The degree of this increase however

varied between the two groups.

All the results from the experiments carried out on nontreated and cp treated mice are combined in Figures 21 and 22. As can be observed, 6 out of 7 experiments with nontreated mice show an increase of TAIg on week 3, whereas only one out of 3 show an increase on week 4. With cp treated mice, 3 out of 4 experiments show an increase of TAIg on weeks 3 and 4.

Figures 23 and 24 include the combined results obtained with nontreated and cp treated mice, presented in the form of mean TAIg binding versus time. While nontreated mice exhibit a fluctuating pattern, cp treated mice show a slow but consistent increase of TAIg binding with time. The variation of tumor size with time in nontreated and cp treated mice together with the variation of TAIg binding is also shown in Figures 23 and 24.

Figure 25 shows the combined results obtained with nontreated and cp treated mice, presented in the form of TAIg binding with size. Tumor sizes $>$ 15 mm in nontreated mice and $<$ 15 mm in cp treated mice are considered since the majority of their respective tumors lie within these boundaries. Whereas only 52% of nontreated mice show a TAIg value above 2.5 ng, 80% of cp treated mice do. Moreover, the in vivo bound TAIg in both groups is positively correlated with size. Figure 26 depicts the variation of TAIg with size in nontreated tumors. A linear relationship can be observed between TAIg binding and size.

It was observed that normal rabbit serum (NRS) bound to the cells obtained from the tumor, though to a much lesser extent than rabbit anti-mouse serum (RAM). However, the variation of NRS binding with time seemed to give a pattern similar to that obtained with RAM.

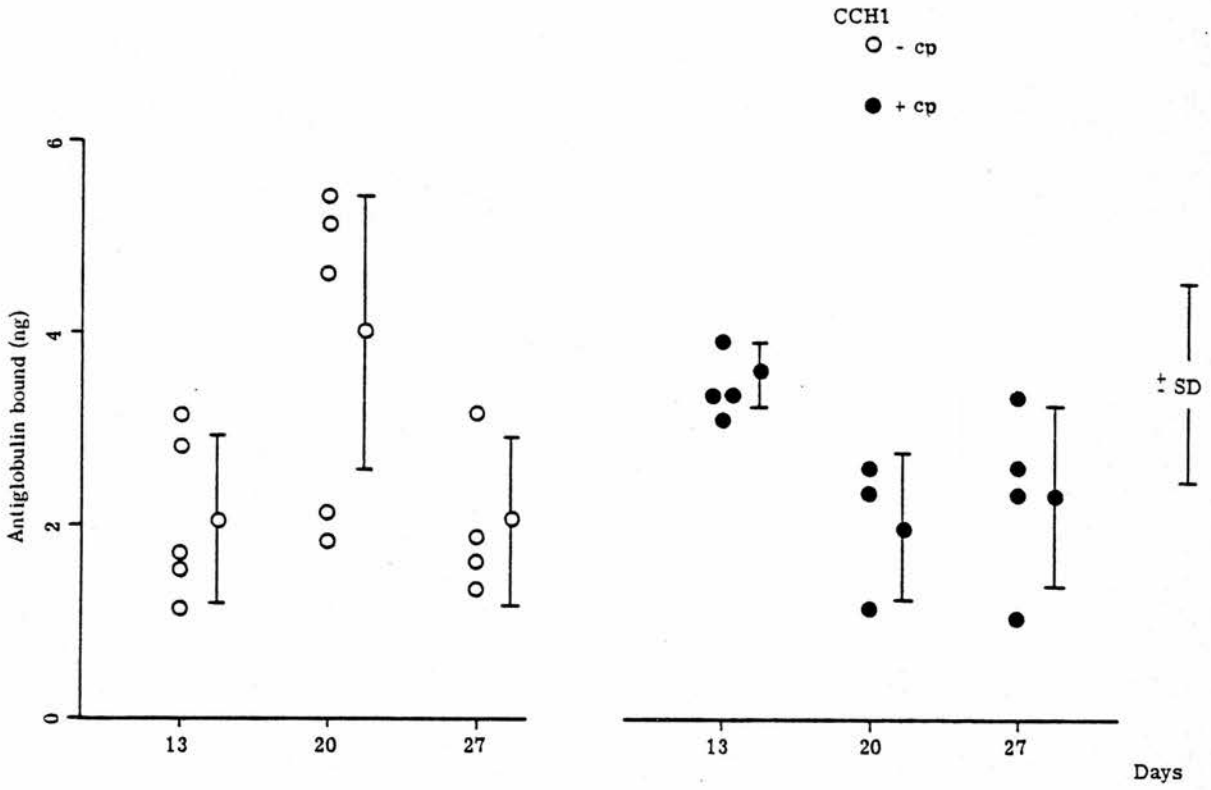


FIG 19 THE VARIATION OF TAIg BINDING WITH TIME IN CCH_1 TUMORS AND THE EFFECT OF C.PARVUM ON THIS VARIATION

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH_1 tumor cells. Half of these were treated ip with 1.4 mg of C. parvum on Day 3. At various times thereafter, the amount of host Ig associated with the surface of 5×10^4 cells from individual tumors was assessed by the indirect radio immune antiglobulin test. It can be observed that TAIg binding increases and decreases during weeks 3 and 4 respectively in nontreated mice. In contrast, TAIg binding decreases and increases during weeks 3 and 4 respectively in C.parvum treated mice. Note that TAIg amounts in C. parvum treated mice are appreciably higher than TAIg amounts in non treated mice on day 13.

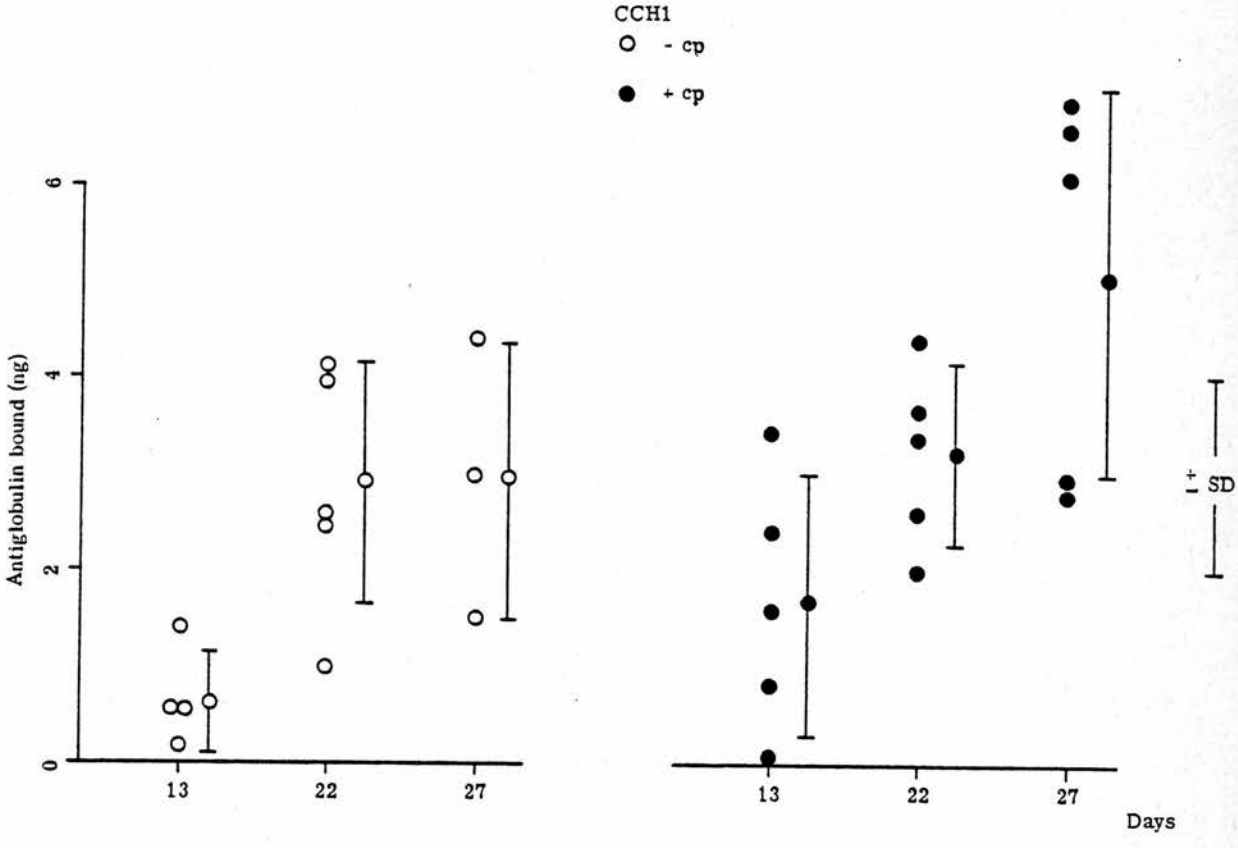


FIG 20 THE VARIATION OF TAIg BINDING WITH TIME IN CCH1 TUMORS, AND THE EFFECT OF C.PARVUM ON THIS VARIATION

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 tumor cells Half of these mice were treated ip with 1.4mg of C. parvum on Day 3. At various times thereafter the amount of host Ig associated with the surface of 5 x 10^4 cells from individual tumors was assessed by the indirect radio immune antiglobulin test. It can be observed that in both C. parvum treated and nontreated mice TAIg binding increases during week 3 of tumor growth. However, while TAIg binding remains the same on day 27 in nontreated mice, it continues to increase in C. parvum treated mice. Note that TAIg amounts on days 13 and 27 in C. parvum treated mice are appreciably higher than TAIg amounts in non-treated mice.

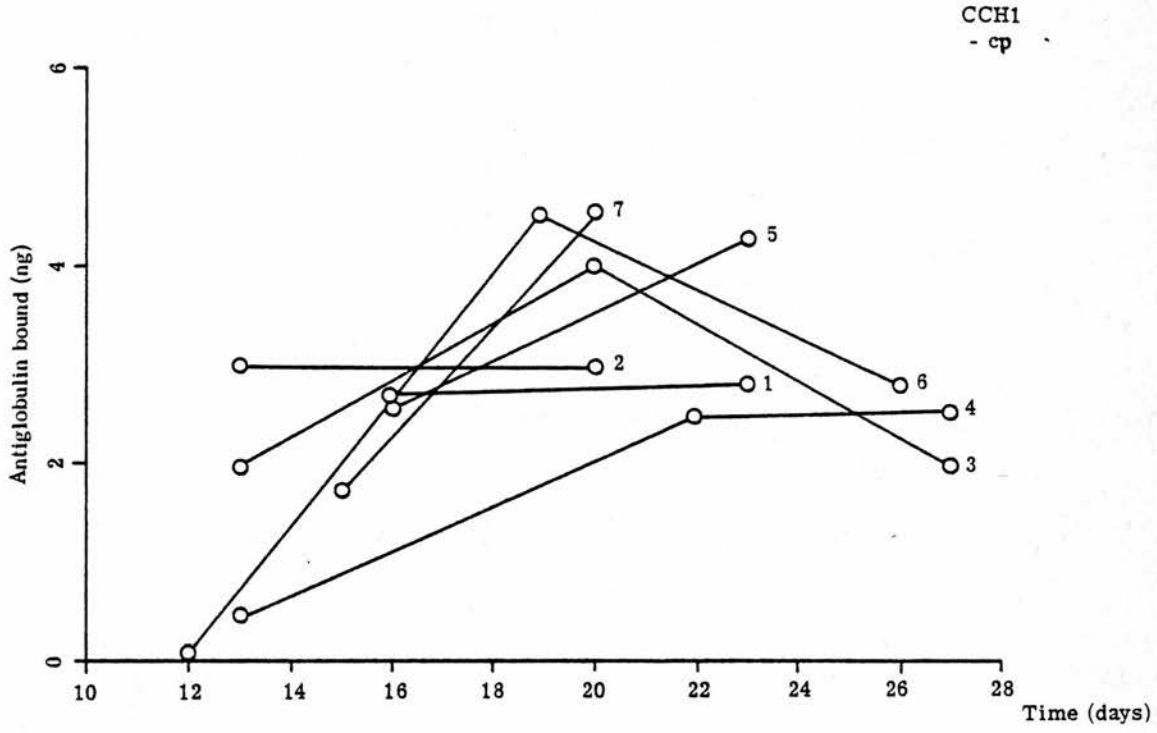


FIG 21 THE VARIATION OF TAIg BINDING WITH TIME IN NON TREATED CCH1 TUMOR-BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 tumor cells. The figure depicts mean TAIg amounts obtained at various time intervals in 7 experiments. Note that in 6 out of the 7 experiments, TAIg binding increases during the 3rd week of tumor growth. In only 3 experiments did the mice survive long enough for their tumors to be studied on the fourth week. Of these 3 experiments two experiments show a drop of TAIg levels during the 4th week of tumor growth.

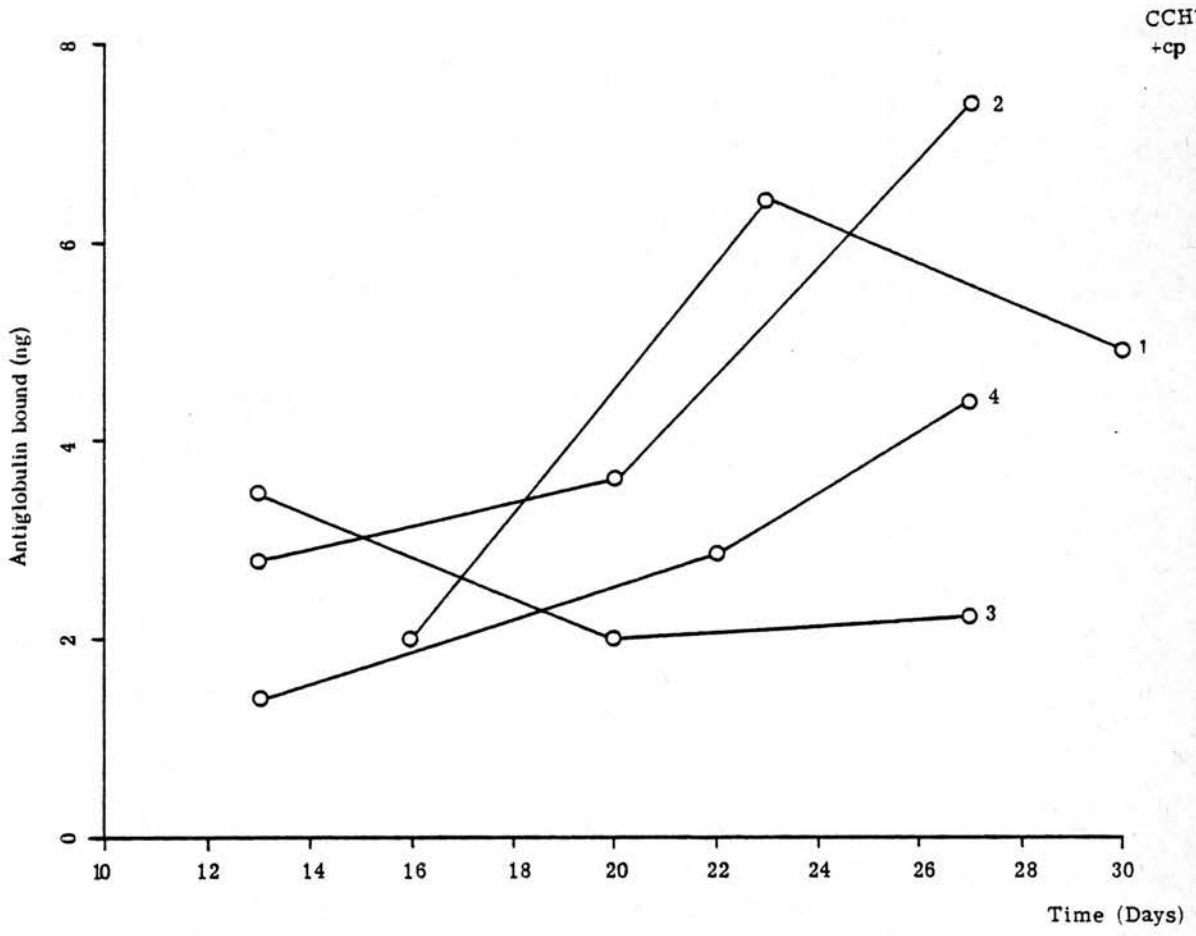


FIG 22 THE VARIATION OF TAIg BINDING WITH TIME IN C. PARVUM TREATED CCH1 TUMOR-BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 tumor cells. On day 3, the mice were injected ip with 1.4 mg of C. parvum. The figure depicts mean TAIg amounts obtained at various time intervals in 4 experiments. Note that in 3 out of the 4 experiments, TAIg binding increases during the 3rd week of tumor growth. Moreover, in contrast to the observation made with nontreated mice (see Fig 21), TAIg binding in C. parvum treated mice increases during the 4th week in 3 out of 4 experiments.

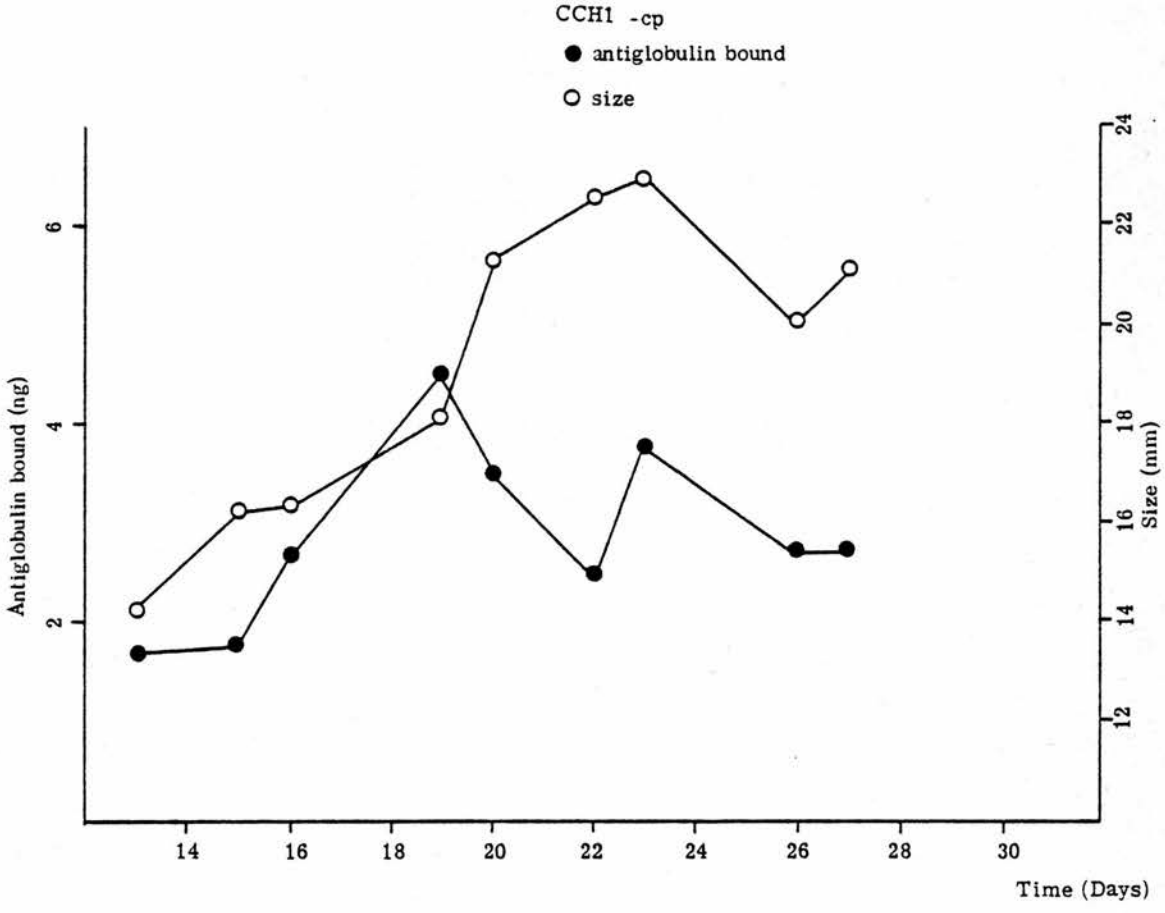


FIG 23 THE VARIATION OF TAIg BINDING AND TUMOR SIZE WITH TIME IN NON-TREATED CCH1 TUMOR BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 tumor cells. The figure depicts mean TAIg amounts and tumor sizes obtained at various time intervals of tumor growth in different experiments. Note that there is consistent increase of TAIg binding and tumor size during the 3rd week of tumor growth. However, while tumor size continues to increase until day 24, TAIg binding appears to fluctuate. Thereafter, tumor size and TAIg binding once again vary similarly with time, decreasing on day 26 and increasing on day 28.

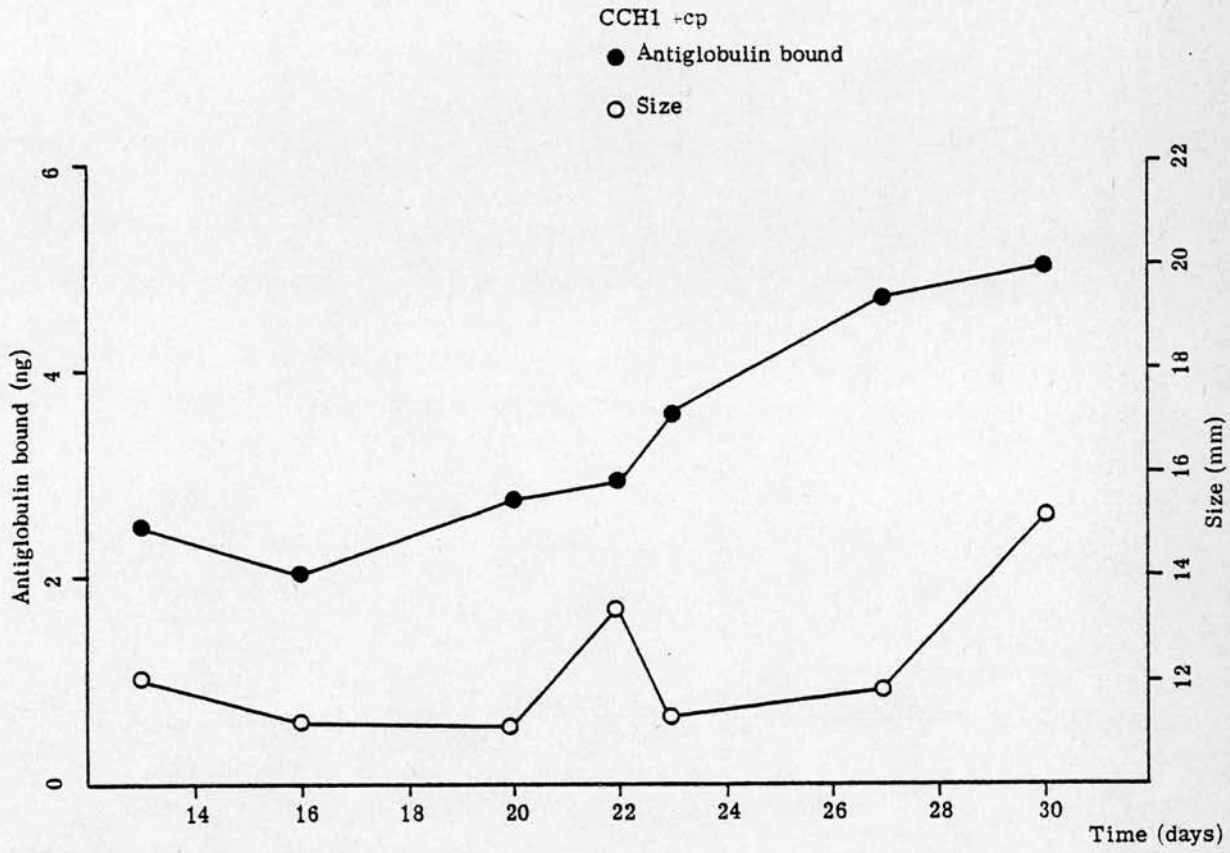


FIG 24 THE VARIATION OF TAIg BINDING AND TUMOR SIZE WITH TIME IN C.PARVUM TREATED CCH1 TUMOR BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 tumor cells. On day 3, the mice were injected ip with 1.4 mg of C. parvum. The figure depicts mean TAIg amounts and tumor sizes obtained at various time intervals of tumor growth in different experiments. Note that there is consistent increase of TAIg binding with time. In contrast, tumor size remains small and begins to increase only during the 4th week of tumor growth.

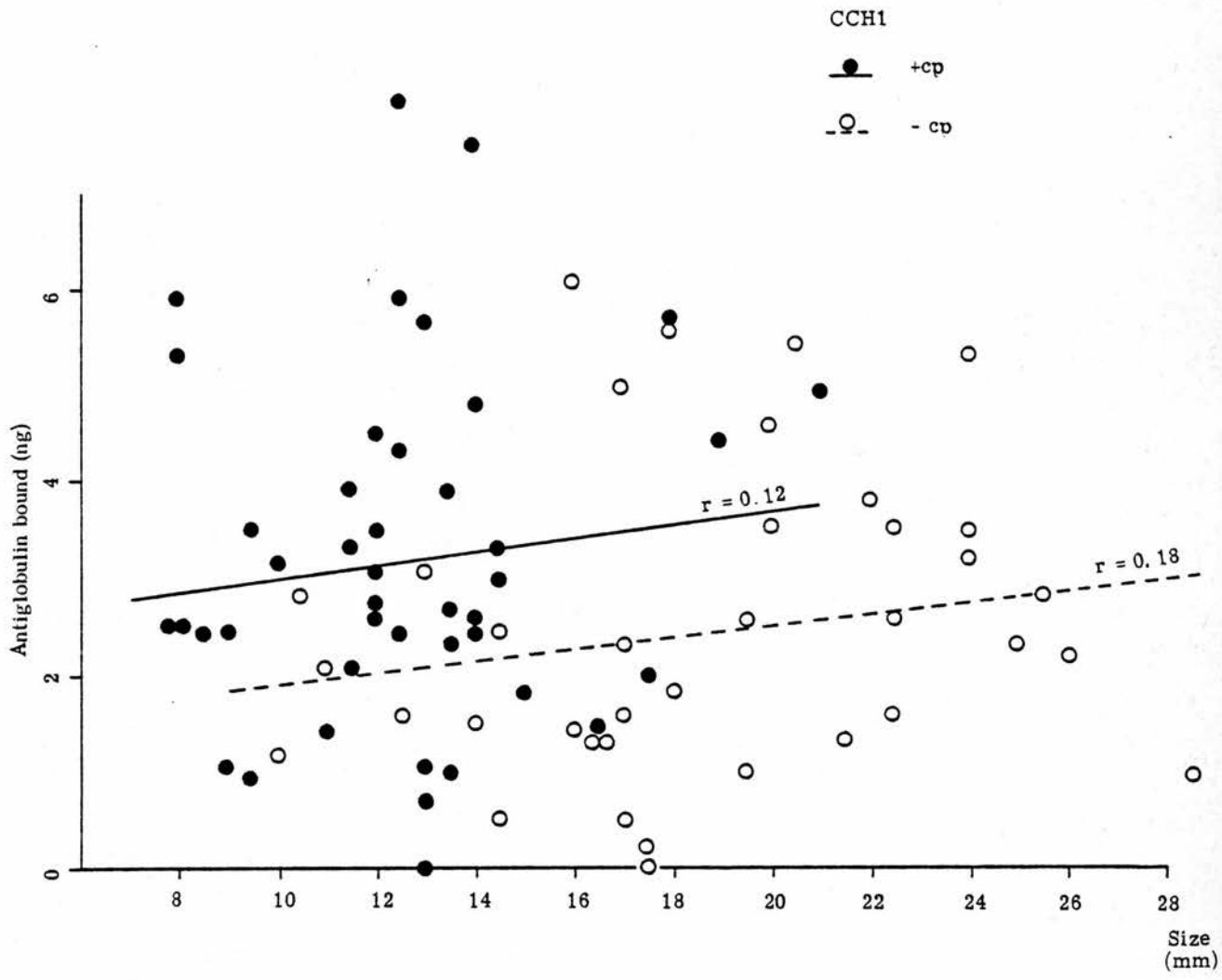


FIG 25 THE VARIATION OF TAIg BINDING WITH TUMOR SIZE IN C. PARVUM TREATED AND NONTREATED CCH1 TUMOR BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 tumor cells. On Day 3, half of the mice were injected ip with 1.4 mg of C. parvum. The figure depicts tumor size and the amount of host Ig associated with the surface of 5×10^4 cells from the corresponding tumors in the four experiments carried out on C. parvum treated and nontreated mice. It can be observed that while the slope of variation of TAIg binding with respect to tumor size does not differ between C. parvum treated and nontreated mice, the former exhibit markedly higher levels of TAIg than the latter in spite of their smaller sizes.

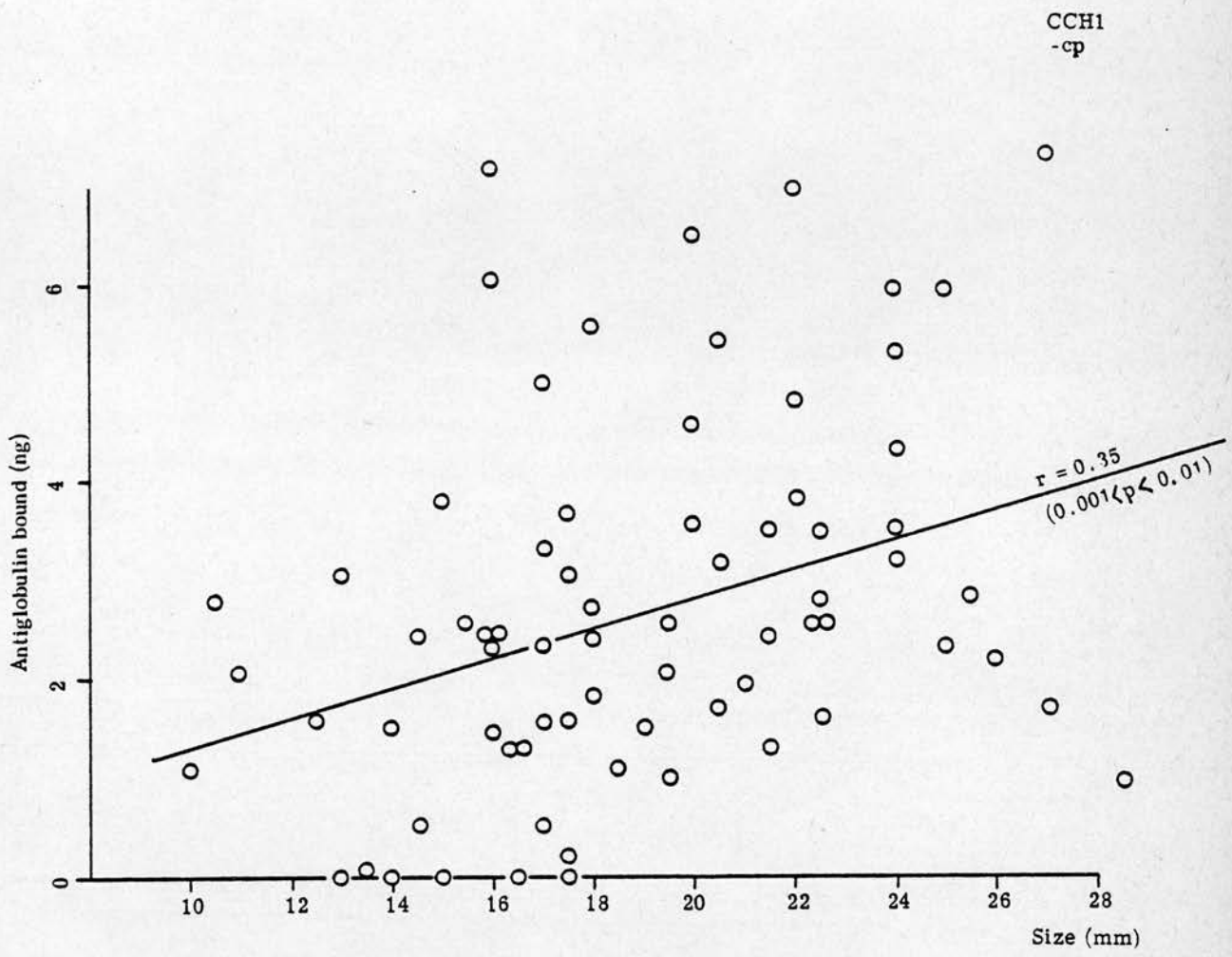


FIG 26 THE VARIATION OF TAIg BINDING WITH SIZE IN NONTREATED CCH1 TUMOR BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 tumor cells. The figure depicts tumor size and the amount of host Ig associated with the surface of 5×10^4 cells from the corresponding tumor in all the experiments carried out on CCH1 tumor bearing mice. It can be observed that there is a significant positive correlation between TAIg binding and tumor size.

THE RESPONSE TO T3 IN NONTREATED AND CP TREATED MICE

The *in vivo* bound TAIg and its variation with time and size of the T3 tumors in nontreated and cp treated mice was compared in 3 experiments. In general, cp treated mice exhibited higher *in vivo* bound TAIg levels, although the pattern of response in relation to time between the 2 groups remained similar.

In the first experiment nontreated mice showed hardly any increase of TAIg on the 3rd week of tumor growth. Cp treated mice, on the other hand, not only showed an increase of TAIg on week 3 but the levels were also higher than those of nontreated mice.

Different batches of ^{125}I GAR were used on week 2 as opposed to weeks 3 and 4 in experiment 2. Whereas nontreated and cp treated mice could be compared on week 2, their TAIg levels could not be compared with those obtained on weeks 3 and 4. On week 2, cp treated mice showed once again higher levels of TAIg compared to nontreated mice. Unlike the case in experiment 1, however, no difference was exhibited between the 2 groups on week 3. The tumors in this experiment were grown from cultured T3 tumor cells.

The results of experiment 3 were similar to those obtained from the previous two experiments in that cp treated mice again consistently showed higher levels of TAIg. However, a slightly different pattern of response was obtained between the 2 groups with relation to time. While nontreated mice showed a decrease of TAIg on week 3 and no variation on week 4, cp treated mice showed a decrease and an increase on weeks 3 and 4 respectively (Fig. 27). The standard deviation in this experiment was less than those observed in the previous 2 experiments.

Figures 28 and 29 combine the results of the 3 experiments in the form of mean in vivo bound TAIg variation with time. Although differences in the pattern of response between the 2 groups is not as apparent as observed with CCH_1 tumors, cp treated mice nevertheless show a more acute variation of TAIg with time compared to nontreated mice. Moreover, tumors obtained from cp treated mice show almost consistently higher amounts of TAIg.

THE RESPONSE TO CCH1 AND T3 TUMORS

The results of the two experiments that were carried out in this group showed the CCH1 cells to be more immunogenic. The latter exhibited higher levels of in vivo bound TAIg in most cases.

Cultured T3 tumor cells were used in the first experiment in order to produce the T3 tumors. The CCH1 tumors were grown from freshly excised CCH1 tumor cells. As can be observed from Figure 30, the CCH1 tumors had higher levels of TAIg on day 13. By the second week of tumor growth however, the TAIg levels in T3 tumors had increased to the same levels of those of CCH1 tumors. In both tumors the in vivo bound TAIg increased with time.

T3 tumors were grown from freshly excised T3 tumor cells in the second experiment where again they exhibited lower TAIg levels than CCH1 tumors which, unlike the first experiment, did not increase with time (Fig. 31).

In general, T3 tumors consistently showed lower levels of TAIg than CCH1 tumors, although the pattern of variations with time did not differ much.

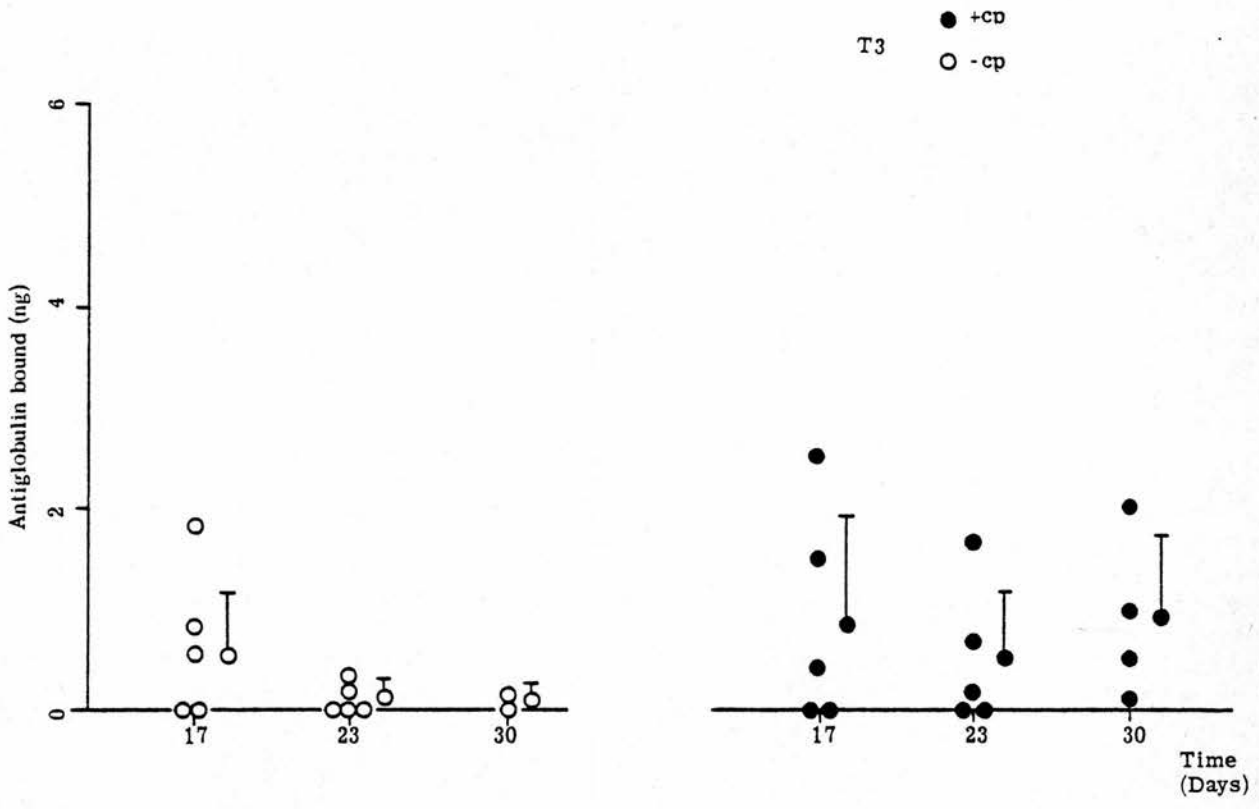


FIG 27 THE VARIATION OF TAIg BINDING WITH TIME IN T3TUMORS, AND THE EFFECT OF C. PARVUM ON THIS VARIATION.

CBA mice were injected s.c. on Day 0 with 10^6 viable T3 tumor cells. On day 3, half of these mice were injected ip with 1.4 mg of C. parvum. At various times thereafter the amount of host Ig associated with the surface of 5×10^4 cells from individual tumors was assessed by the indirect radio immune antiglobulin test. It can be observed that TAIg binding decreases after the 3rd week in nontreated mice. In contrast, after an initial drop during the 3rd week, TAIg binding increases in C.parvum treated mice. In addition, tumors from C. parvum treated mice exhibit higher amounts of TAIg than tumors from nontreated mice at all the times tested.

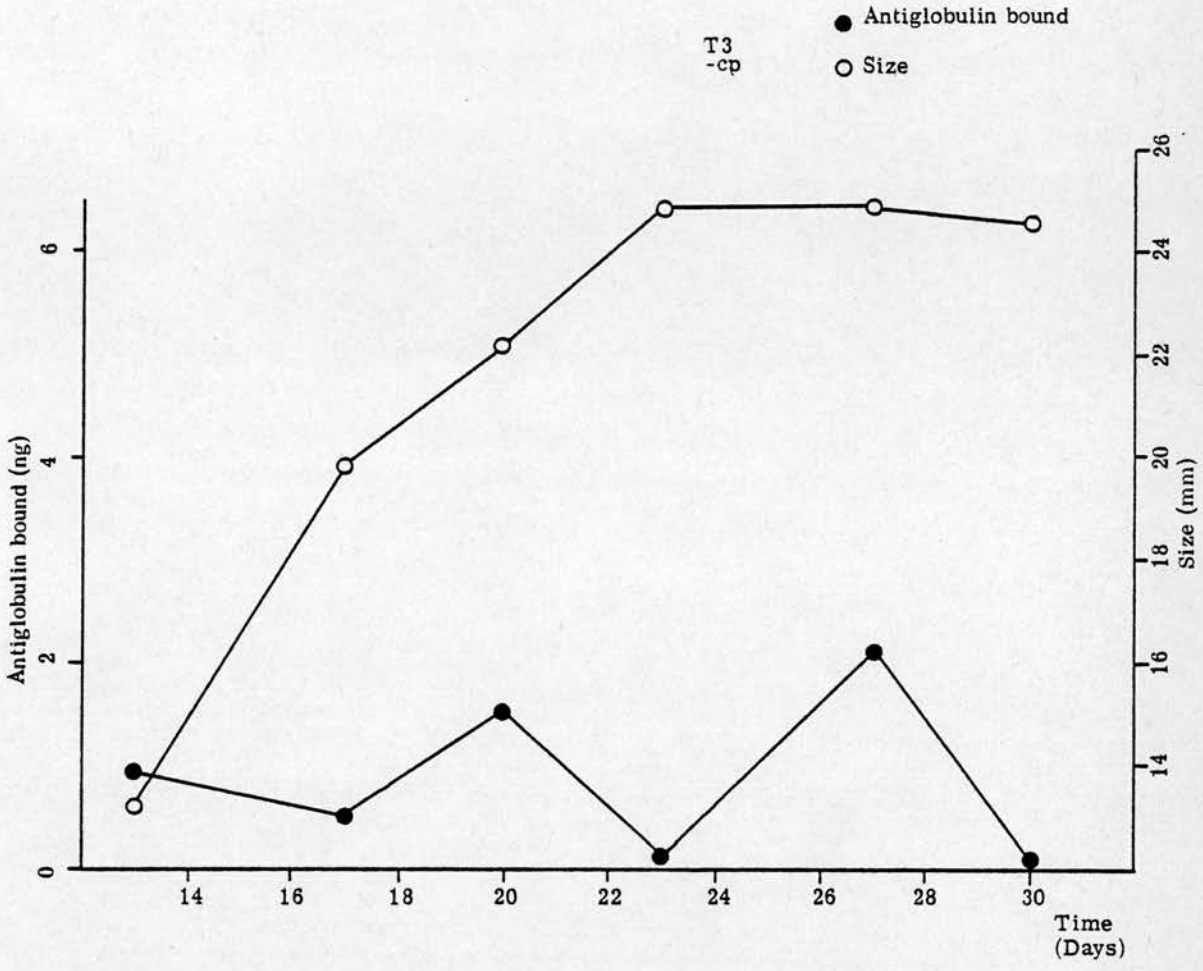


FIG 28 THE VARIATION OF TAIg BINDING AND TUMOR SIZE WITH TIME IN NONTREATED T3 TUMOR BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable T3 tumor cells. The figure depicts mean TAIg amounts and tumor sizes obtained at various time intervals of tumor growth in different experiments. Note that while there is a consistent rapid increase of tumor size with time, there is a fluctuating variation of TAIg binding with time.

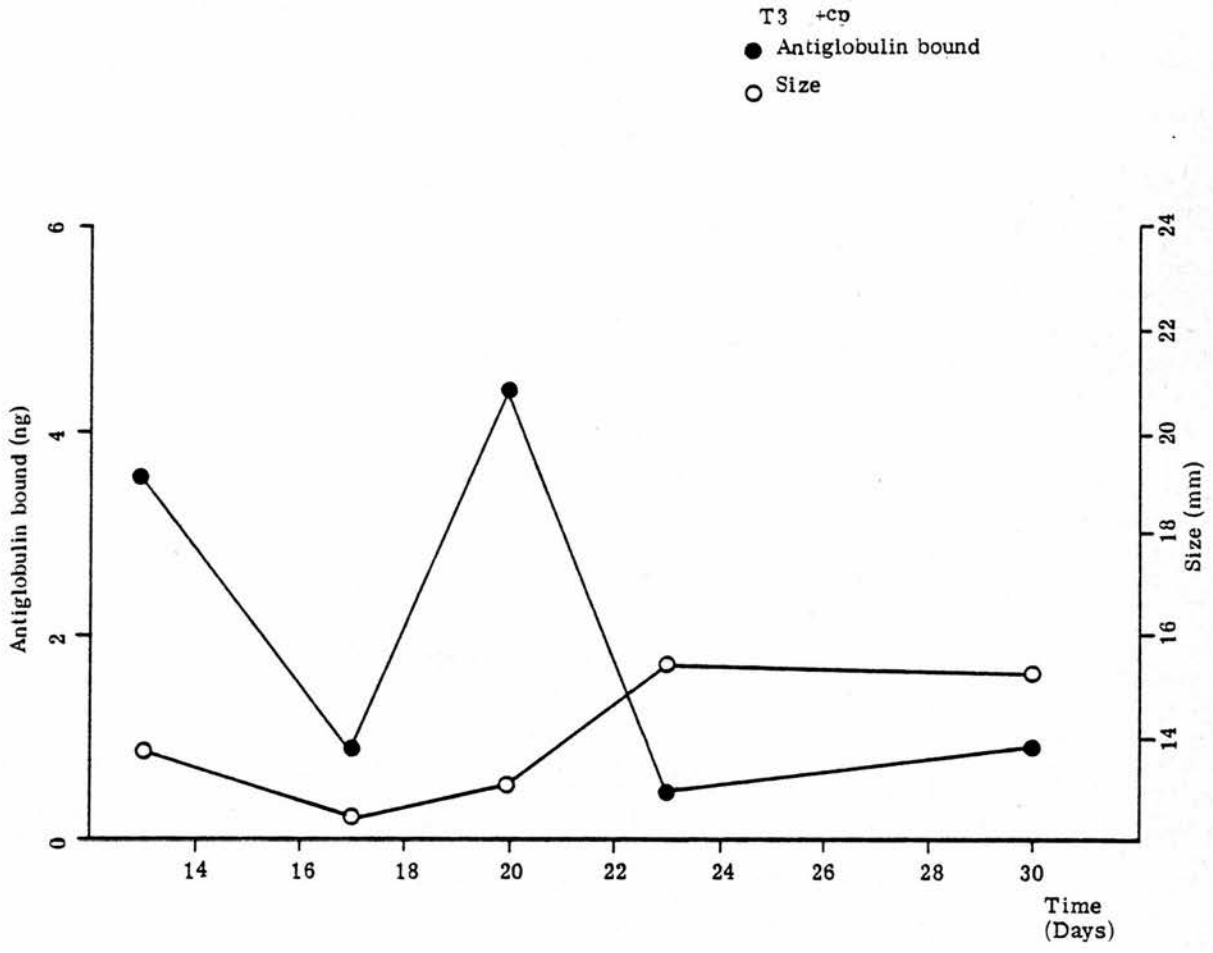


FIG 29 THE VARIATION OF TAIg BINDING AND TUMOR SIZE WITH TIME IN C. PARVUM TREATED T3 TUMOR BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable T3 tumor cells. On day 3, the mice were injected ip with 1.4 mg of C. parvum. The figure depicts mean TAIg amounts and tumor sizes obtained at various time intervals of tumor growth in different experiments. Note that the fluctuating variation of TAIg binding with time obtained in nontreated mice (see Fig 28) is again observed with C.parvum treated mice. However, the amounts of TAIg in C. parvum treated mice are appreciably higher than nontreated mice. Note also that tumor growth is markedly inhibited in C.parvum treated mice.

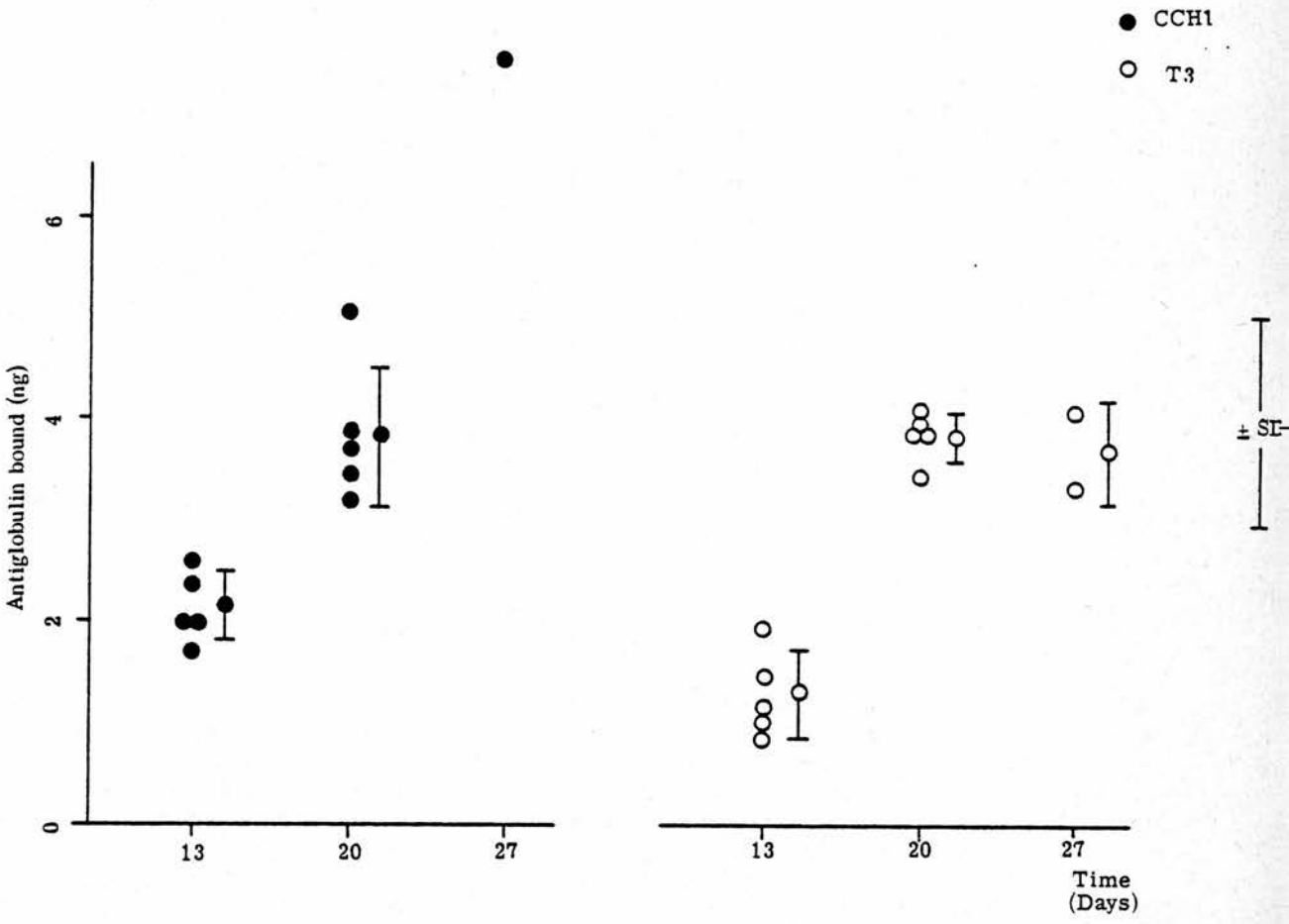


FIG 30 THE VARIATION OF TAIg BINDING WITH TIME IN CCH1 AND T3 TUMOR BEARING MICE

Equal numbers of CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 or T3 tumor cells. At various times thereafter the amount of host Ig associated with 5×10^4 cells from individual tumors was assessed by the indirect radio immune antiglobulin test. It can be observed that TAIg increases during the 3rd week in both CCH1 and T3 tumors. However, the amount of TAIg on day 13 is lower in T3 tumors than CCH1 tumors. Note that there is no increase of TAIg binding in T3 tumors during week 4.

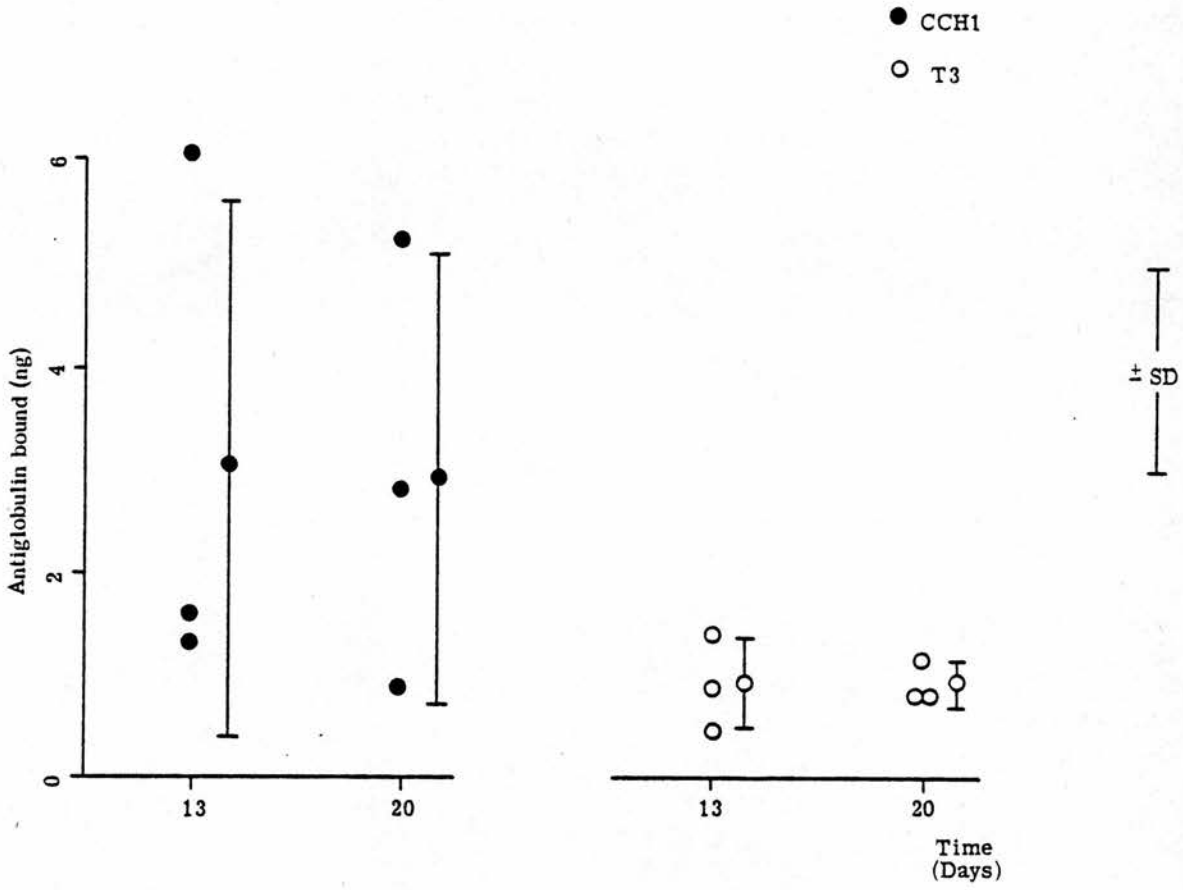


FIG 31 THE VARIATION OF TAIg BINDING WITH TIME IN CCH1 AND T3 TUMOR BEARING MICE

Equal numbers of CBA mice were injected s.c on Day 0 with 10^6 viable CCH1 or T3 tumor cells. On days 13 and 20 of tumor growth the amount of host Ig associated with 5 x 10^4 cells from individual tumors was assessed by the indirect radio immune antiglobulin test. No variation of TAIg binding with time was observed in both CCH1 and T3 tumor bearing mice.

THE RESPONSE TO CCH1 EXCISED AND CULTURED TUMOR CELLS

Two experiments were carried out to study the *in vivo* bound TAIg variations with age and size of tumors induced by excised and cultured CCH1 cells. Tumors induced by cultured cells exhibited lower levels of TAIg and smaller tumor sizes.

In the first experiment, on days 16 and 23 of tumor growth, tumor produced by excised CCH1 cells showed higher levels of TAIg than those produced by cultured cells. Moreover, the TAIg in tumors produced by excised CCH1 cells increased in the third week, while those of tumors produced by cultured CCH1 cells remained constant on the 3rd week, increasing substantially only in the 4th week.

The results obtained in the 2nd experiment were consistent with those of the first experiment with respect to TAIg levels. Tumors produced by excised CCH1 cells again showed higher TAIg levels than those produced by cultured cells. However, the TAIg level in this experiment decreased in the 4th week of tumor growth (Fig. 32).

Tumors produced by cultured cells were not only smaller in size, but also the great majority of them had TAIg levels lower than 2 ng. Conversely, tumors produced by excised CCH1 cells had larger sizes as well as higher TAIg levels.

THE RESPONSE TO DIFFERENT DOSES OF CCH1 TUMOR CELLS

The response to 2 different doses of 0.5 and 5 x 10^6/ml CCH1 cells was studied in one experiment. Tumors produced by the larger dose showed higher levels of TAIg and were of larger sizes (Fig. 33).

The TAIg levels in both groups increased with time. Moreover, 60% of tumors produced by the larger dose exhibited levels of TAIg above 2 ng, compared to only 10% of those produced by the smaller dose.

THE RESPONSE TO PRONASE PREPARED AND MECHANICALLY PREPARED CCH1 CELLS

Mice were injected with pronase prepared or mechanically prepared CCH1 cells consisting of equal numbers of viable cells (1×10^6/ml). Pronase prepared cells produced larger tumors with higher TAIg levels (Fig. 34). Once again, the TAIg in both groups increased with age and size of tumors.

SUMMARY

The general pattern observed is that of an increase of *in vivo* TAIg binding with age and size of CCH[1] and T3 tumors of nontreated mice. Although the levels of TAIg in CCH1 and T3 tumors differ, their pattern of variation with time and size of the tumors seem to be similar.

With cp treated mice a similar positive correlation of TAIg binding with age and size of tumors is obtained. But while in nontreated mice only large tumors tend to exhibit high levels of TAIg, in cp treated mice the high levels are observed in small tumors. Moreover, the pattern of variation of TAIg with time is different in CCH1 and T3 tumors. Whereas in CCH1 tumors the TAIg increases with time, in T3 tumors it seems to fluctuate

Tumors produced by excised CCH1 cells exhibit higher levels of TAIg than those produced by cultured CCH1 cells. In addition, the latter seem to grow slower than excised CCH1 cells, resulting in smaller tumors.

In vivo TAIg binding seems to be dose dependent, with larger doses resulting in higher binding.

Finally, tumors produced by mechanically prepared cells are smaller in size and exhibit lower TAIg levels than those produced by pronase prepared cells.

In conclusion, in vivo TAIg binding in nontreated mice appears to be dose, cell-type and cell preparation mode dependent. Moreover, high levels of TAIg seem to correlate with cp tumor growth inhibition.

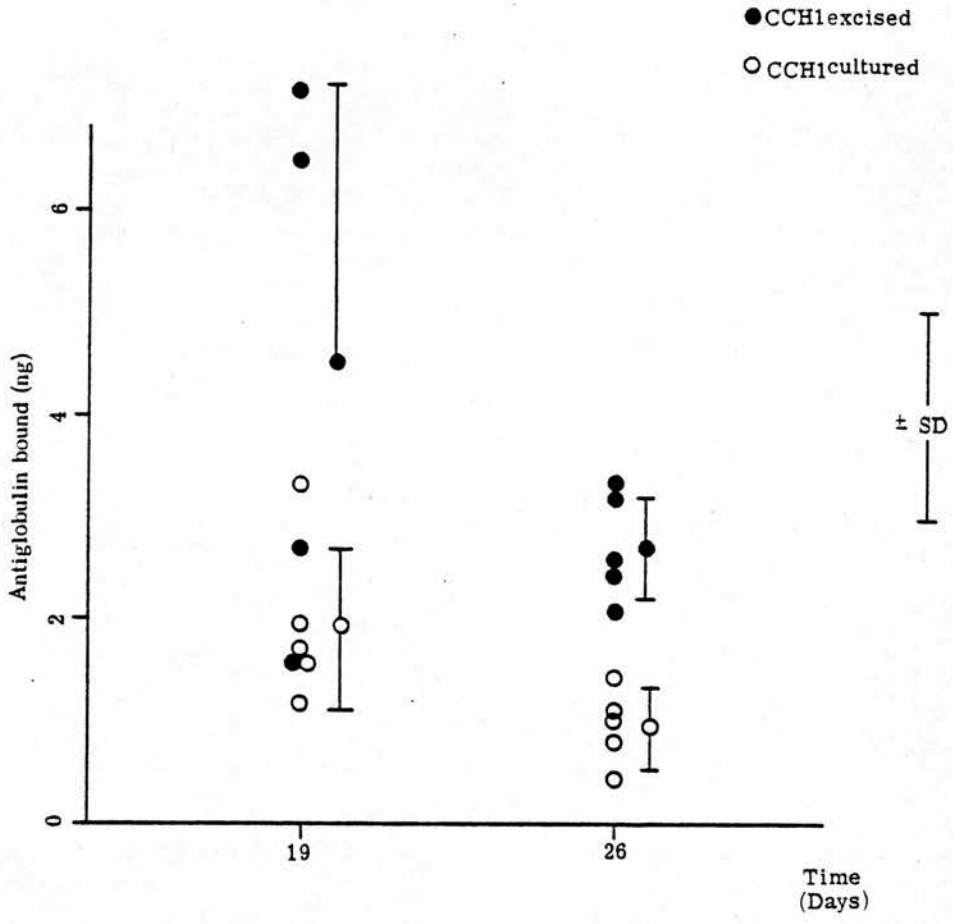


FIG 32 THE VARIATION OF TAIg BINDING WITH TIME IN CCH1 FRESHLY EXCISED AND CULTURED TUMOR BEARING MICE

Equal numbers of CBA mice were injected s.c. on Day 0 with 10^6 viable freshly excised or cultured CCH 1 tumor cells. On days 19 and 26, the amount of host Ig associated with 5 x 10^4 cells from individual tumors was assessed by the indirect radio immune antiglobulin test. Note that on both days, TAIg amounts are higher in tumors grown from freshly excised tumor cells than tumors grown from cultured tumor cells. In addition, there is a decrease of TAIg binding in both tumor groups during the 4th week of tumor growth.

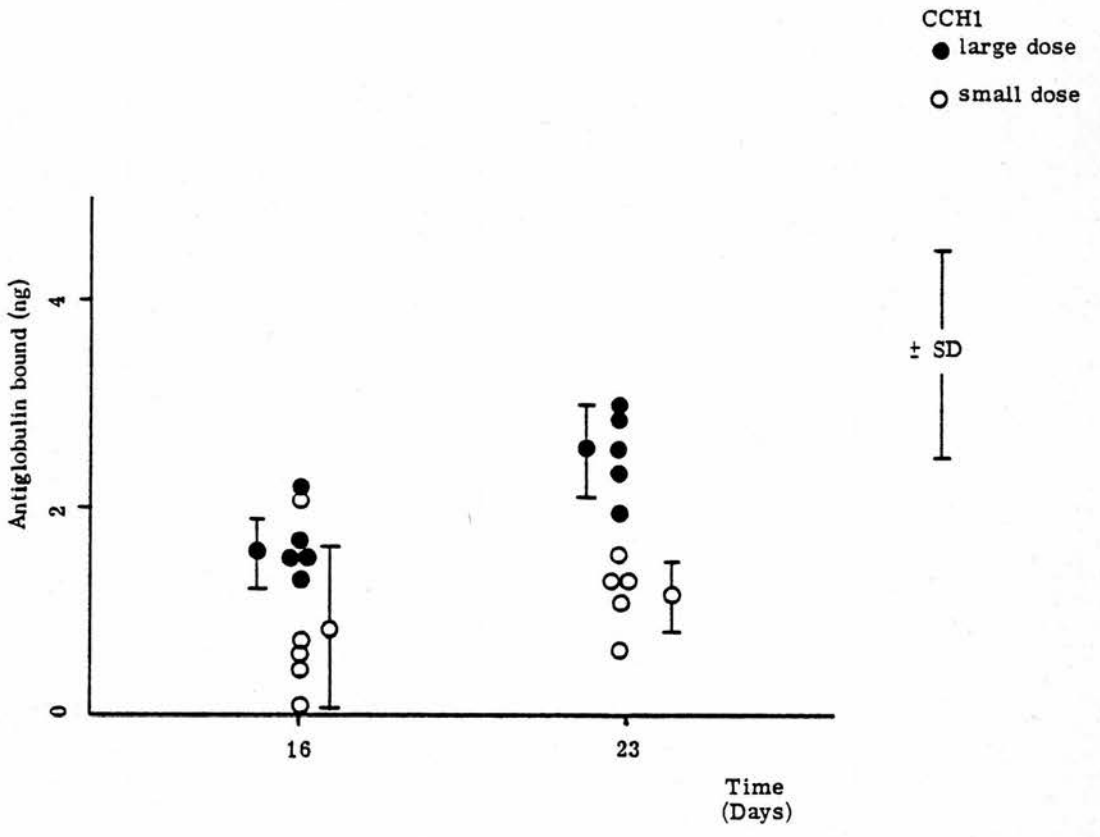


FIG 33 THE EFFECT OF DOSE ON TAIg BINDING IN CCH1 TUMORS

Equal numbers of CBA mice were injected s.c. on Day 0 with 0.5 and 5 x 10^6 viable freshly excised CCH1 tumor cells respectively. On days 16 and 23, the amount of host Ig associated with 5 x 10^4 cells from individual tumors was assessed by the indirect radio immune antiglobulin test. It can be observed that on both days, tumors produced by the larger dose of tumor cells exhibit higher amounts of TAIg than tumors produced by the smaller dose of tumor cells. Moreover, both groups of tumors exhibit an increase of TAIg binding during the 3rd week of tumor growth.

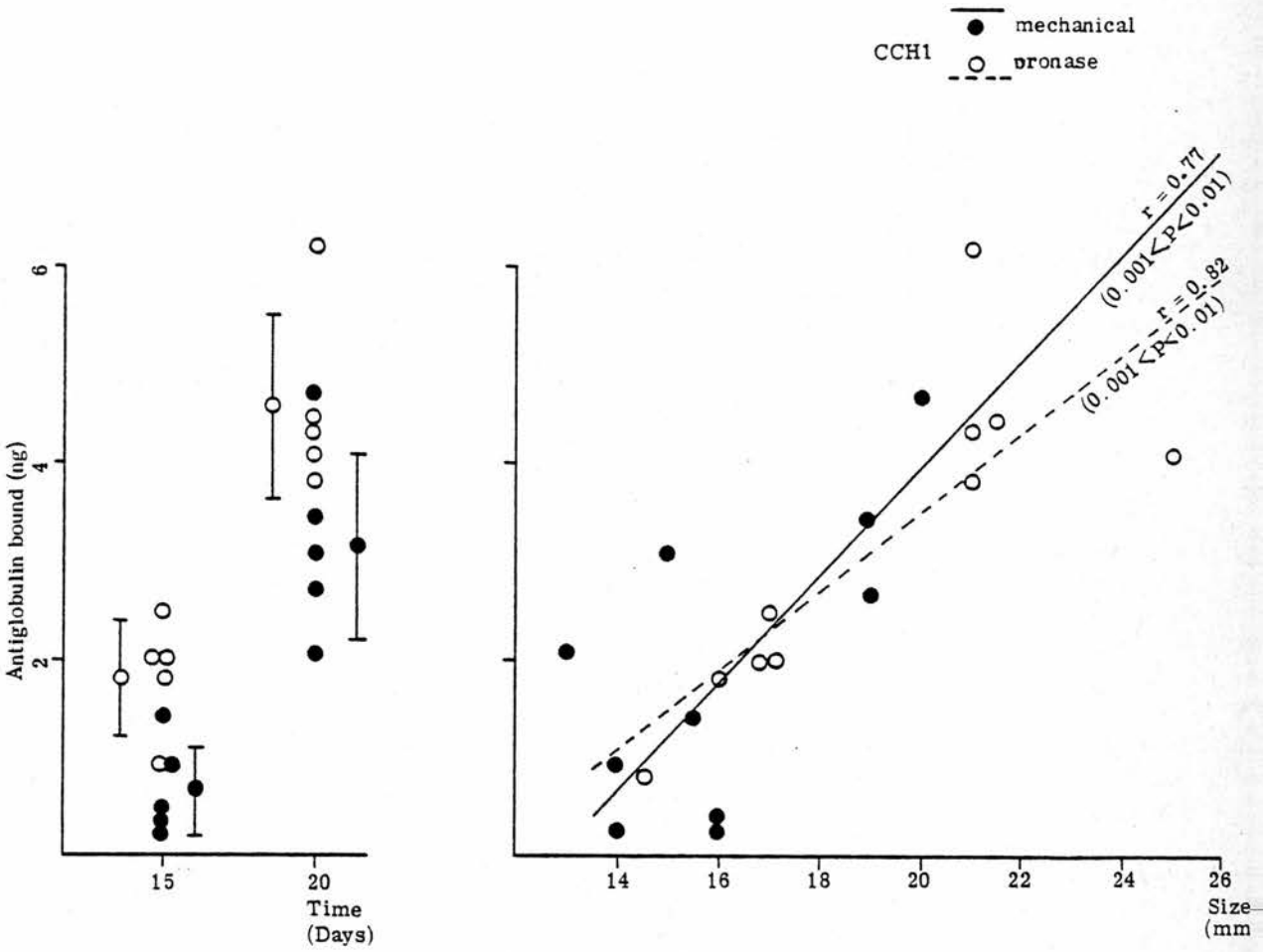


FIG 34 LEFT: THE VARIATION OF TAIg BINDING WITH TIME IN CCH1 FRESHLY EXCISED MECHANICALLY PREPARED AND PRONASE PREPARED TUMOR BEARING MICE

Equal numbers of CBA mice were injected s.c. on Day 0 with 10^6 viable freshly excised CCH1 mechanically prepared or Pronase prepared tumor cells. On days 15 and 20, the amount of host Ig associated with 5 x 10^4 cells from individual tumors was assessed by the indirect radio immune antiglobulin test. Note that on both days, TAIg amounts were higher in tumors grown from pronase prepared cells than tumors grown from mechanically prepared cells. In addition, both groups of tumors exhibit an increase of TAIg binding during the 3rd week of tumor growth.

RIGHT: THE VARIATION OF TAIg BINDING WITH SIZE IN THE CCH1 FRESHLY EXCISED MECHANICALLY PREPARED AND PRONASE PREPARED TUMOR BEARING MICE

Note that in both tumor groups, TAIg binding significantly increases with tumor size

THE DEVELOPMENT OF THE DIRECT RADIOIMMUNE ANTIGLOBULIN TEST

This section describes the experiments which were carried out to determine the optimum conditions for the direct radioimmune antiglobulin test (RIAT) that was to be used for the reassessment of the results obtained by the indirect RIAT and for additional tumor-associated immunoglobulin (TAIg) studies. The standard method which was eventually adopted consisted of incubating 5×10^4 cells with ^{125}I-RAM Ig for 1 hour at $4^{\circ}C$, and then washing the cells 4 times with 5% FCS PBS (further details in Materials and Methods).

The various parameters studied in the development of the direct RIAT included the following :

a. effect of tube precoating with FCS on the reduction of nonspecific binding to plastic.
b. a study of the specificity of the RAM Ig.
c. effect of enzyme treatment on surface bound TAIg.
d. influence of reagent concentration on its binding to tumor cells.
e. effect of incubation temperature and time on the RAM Ig binding to tumor cells.
f. effect of cell number on reagent binding.
g. nonspecific binding of the RAM Ig to dead tumor cells.
h. nonspecific binding to cells via the Fc-portion of the RAM Ig.

The specific reasons behind these studies are mentioned in the introduction to the development of the indirect RIAT.

The appreciable work carried out in this section by my colleague James Merriman is thankfully acknowledged.

EFFECT OF TUBE PRECOATING

In previous studies with the indirect RIAT, overnight precoating of the tubes with 5% FCS at 4°C was found to inhibit nonspecific binding to plastic. In order to assess the effect of FCS on nonspecific binding in the direct method, precoating with the following amounts and volumes of FCS were compared : 0.1 ml neat, 1 ml neat, 4 ml neat, 4 ml 1:5, 4 ml 1:25, and 4 ml 1:125. The concentration of RAM Ig was 4 µg/100 µl.

All the amounts and volumes of FCS studied showed a significant reduction of nonspecific binding to plastic (Figure 35). It was therefore decided to continue the standard procedure of 5% FCS precoating overnight at 4°C.

SPECIFICITY OF THE RAM Ig

The specificity of RAM Ig binding was initially tested on normal spleen cells, normal thymus cells, cultured CCH1 tumor cells and excised CCH1 and CCH5 tumor cells. Two cell concentrations of 7×10^6/ml and 7×10^5/ml were used. As shown in Figure 36, the highest RAM Ig binding was found to be on CCH5 tumor cells, followed by CCH1 tumor cells and spleen cells. The lower-binding of the reagent to the spleen cells may be partially attributed to the smaller size of these cells compared to the tumor cells. Only negligible RAM Ig binding to thymus cells and cultured CCH1 tumor cells was observed.

In a second experiment, spleen cells were used to assess the binding of RAM Ig to surface bound immunoglobulins. Reagent concentrations of 1000, 500, 250 and 125 ng/0.1 ml were used on 1×10^6 and 1×10^5 cells. The

assay was carried out at incubation temperatures of 4 and 37°C. RAM Ig binding to the spleen cells was found to vary directly with cell and reagent concentration (Fig. 37) and the reaction was observed to be enhanced at 37°C.

In a third experiment CCH1 cultured tumor cells were incubated with various dilutions of mouse serum (1:5, 1:10, 1:20, 1:40) for 1 hour at 37°C. The sera were obtained from nontreated and C. parvum treated tumor bearing mice. The cells were then washed 3 times with 4 ml of 10% FCS-PBS and once with 4 ml complete RPMI, and recovered after each wash by centrifugation at 283 g for 10 minutes. The cells were reincubated for a further hour with 50 µl of ^{125}I-RAM Ig at 37°C. The cells were then washed 4 times with 4 ml 10% FCS-PBS, transferred to new tubes and counted. A direct correlation of RAM Ig binding to the cultured cells with serum and cell concentration was observed (Fig. 38). Moreover, cells incubated with sera obtained from C. parvum treated tumor bearing mice exhibited higher binding of the RAM Ig.

EFFECT OF ENZYME TREATMENT

Six experiments were carried out in order to study the effect of enzymes on surface bound TAIg. Similar to previous studies obtained with the indirect method, all the experiments indicated a direct relationship between loss of surface bound TAIg and the time and concentration of enzyme treatment.

In the first experiment, mechanically prepared tumor cells were compared with pronase prepared cells. The latter involved two 5 minute incubations of the cells with the enzyme at 37°C (enzyme conc. 2.5 mg/ml).

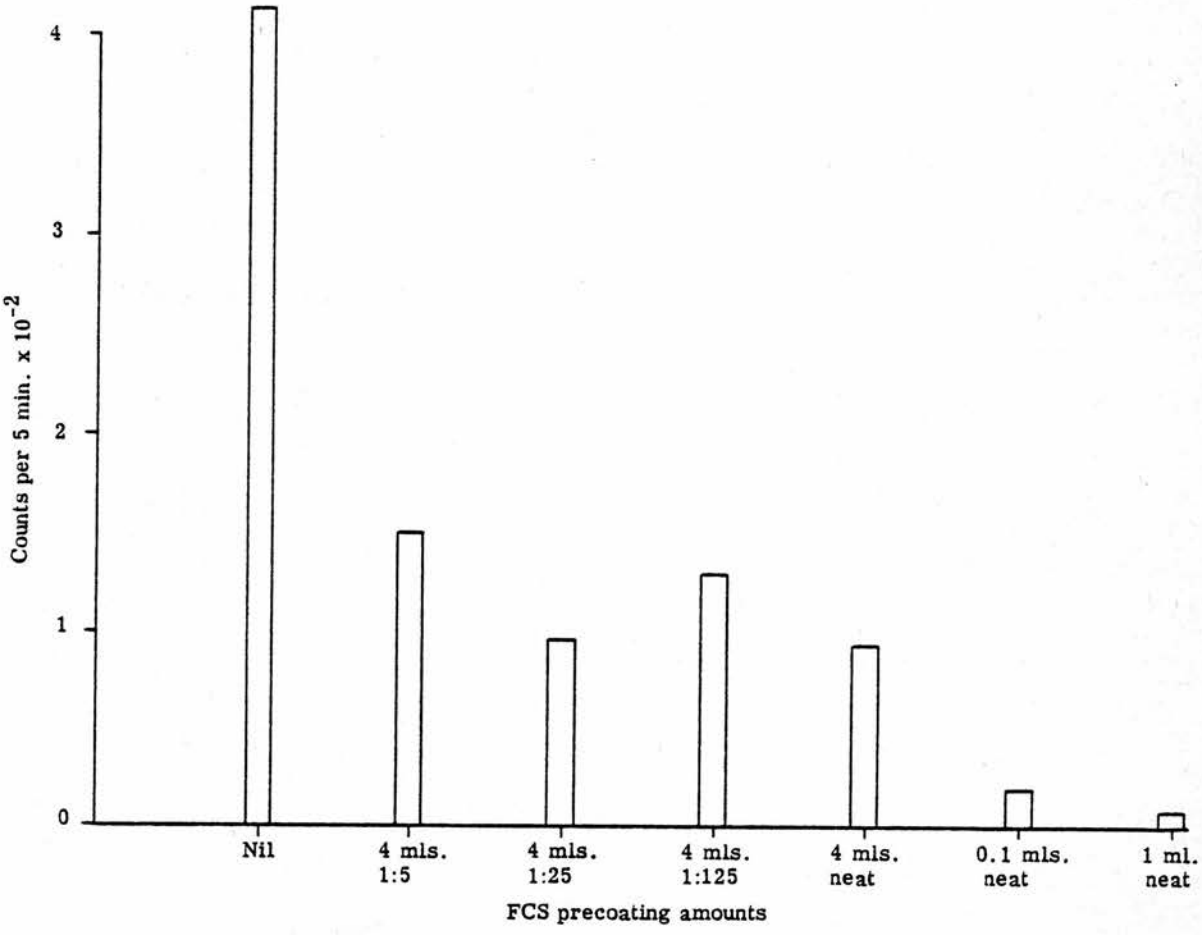


FIG 35 THE EFFECT OF PRECOATING THE TUBES WITH VARIOUS FCS CONCENTRATIONS ON THE NONSPECIFIC BINDING OF REAGENTS TO PLASTIC

Tubes were precoated with PBS consisting of various FCS concentrations overnight at 4°C. The solutions were then decanted and the direct radio immune antiglobulin test carried out. It can be observed that precoating the tubes with various FCS concentrations appreciably inhibited the non specific binding of reagents to plastic.

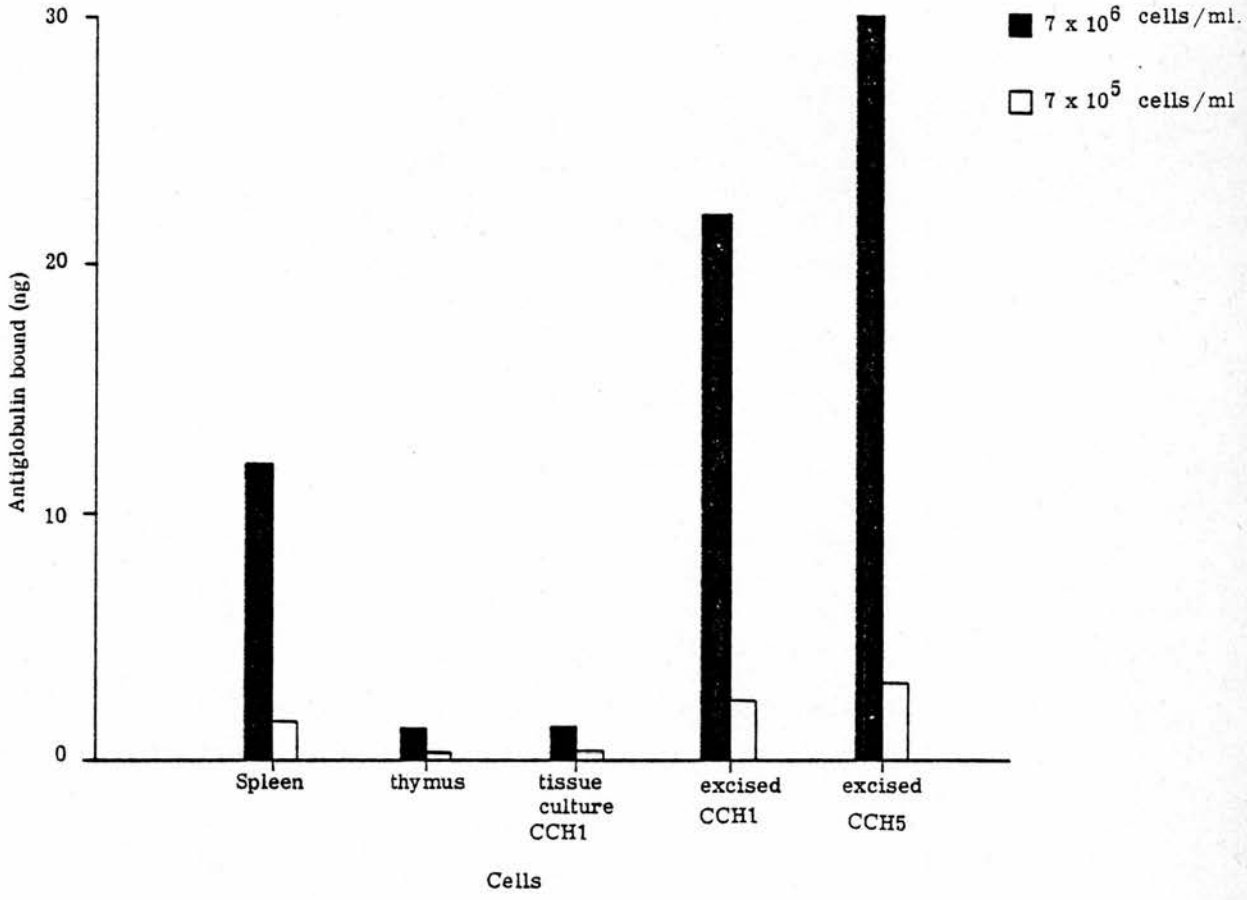


FIG 36 SPECIFICITY OF THE ^{125}I-RAM Ig

In this experiment, the specificity of the RAM Ig to surface-bound immunoglobulins was studied. The binding of the RAM Ig to freshly excised tumor cells and spleen cells (positive cells) was compared with its binding to cultured tumor cells and thymus cells (control cells). Note that a) the RAM Ig binds most to freshly excised tumor cells and spleen cells; b) the binding to cultured tumor cells and thymus cells hardly exceeds background levels c) the binding of the reagent increases with cell numbers.

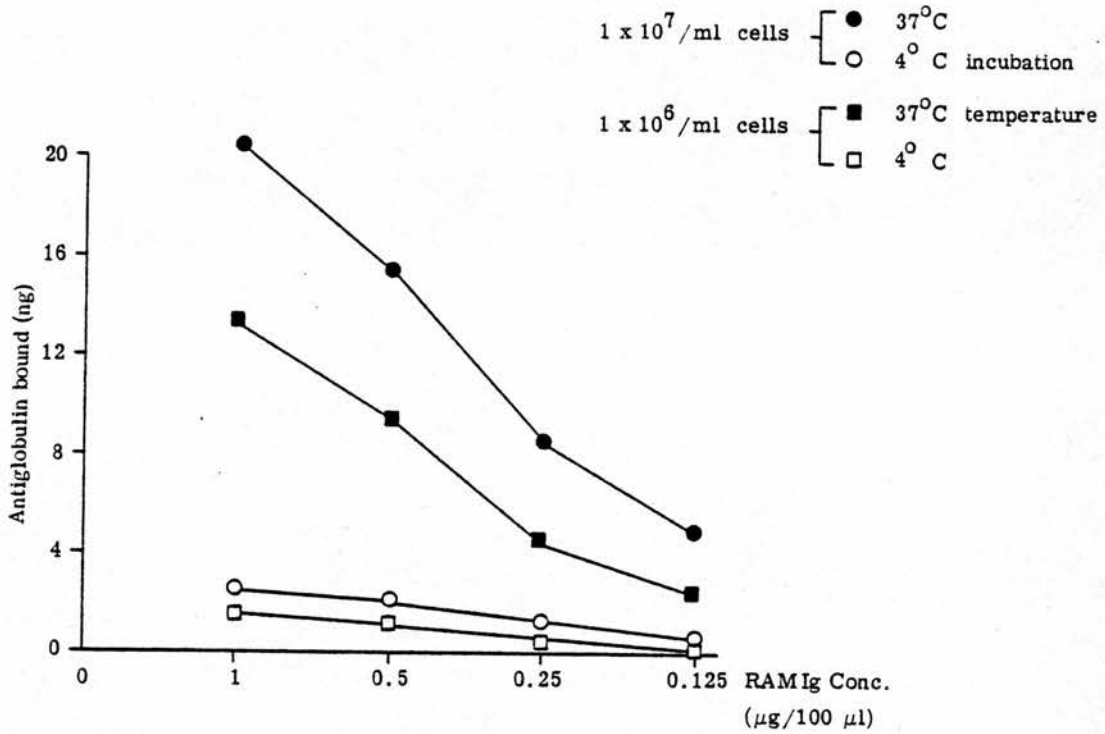


FIG 37 SPECIFICITY OF THE ^{125}I-RAM Ig AND THE VARIATION OF ITS BINDING WITH REAGENT CONCENTRATION AND CELL NUMBER

In this experiment, the specificity of the RAM Ig to surface bound immunoglobulins on spleen cells was studied. Moreover, the variation of reagent binding with its concentration, cell number, and incubation temperature was examined. It can be observed that at both cell numbers and incubation temperatures, RAM Ig binding to the spleen cells varies positively with the reagent concentration. Note that reagent binding to cells also varies positively with cell number and incubation temperature.

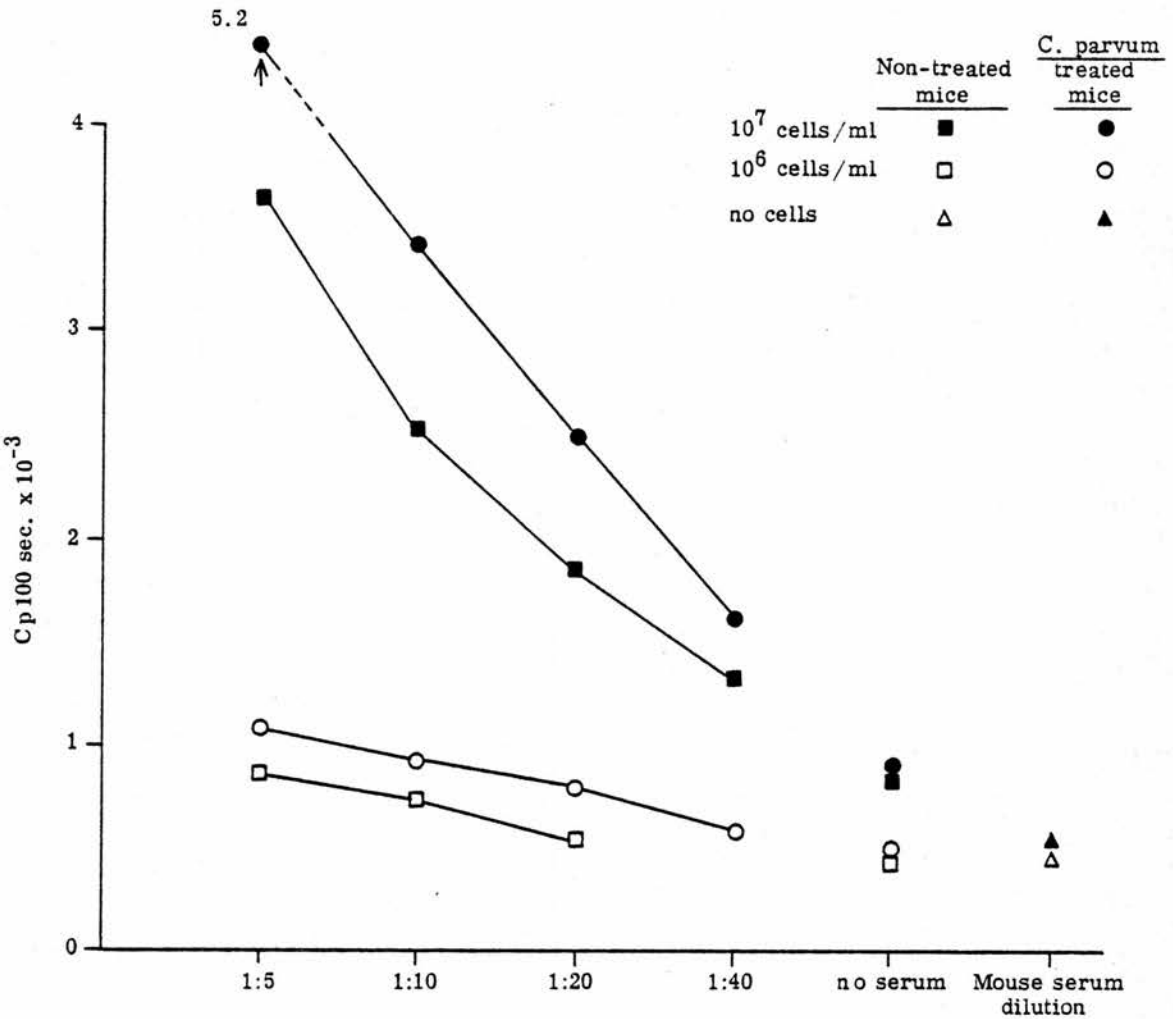


FIG 38 THE SPECIFICITY OF THE ^{125}I-RAM Ig AND THE VARIATION OF ITS BINDING WITH MOUSE SERUM DILUTION AND TUMOR CELL NUMBER

In this experiment, the specificity of the RAM Ig to surface-bound immunoglobulins was studied by examining its binding to cultured tumor cells treated with various dilutions of mouse sera. The latter were obtained from C.parvum treated or non-treated CCH1 tumor bearing mice. It can be observed that RAM-Ig binding to the cells varied positively with mouse serum concentration at both cell numbers. Note that a) reagent binding increases with cell number and b) reagent binding to cells treated with serum from C.parvum treated mice is higher than its binding to cells treated with serum from nontreated mice.

Cell numbers of 2 x 10^6, 10^6, 10^5, 10^4 and 10^3/0.1 ml were used. At all cell concentrations, mechanically prepared cells exhibited a higher amount of surface bound TAIg (Fig. 39). The RAM Ig binding to the cells was found to be enhanced at 37°C.

The effect of prolonged enzyme treatment on TAIg was studied in the second experiment. Periods of the enzyme treatment included 5, 30 and 60 minute incubations at 37°C. As shown in Figure 40, loss of TAIg from tumor cell surface varied directly with time of enzyme treatment. The RAM Ig binding to tumor cells was again found to be enhanced at 37°C.

In the third experiment, pronase and/or collagenase preparation of tumor cells was compared with mechanical preparation. Enzyme preparation of the cells involved the incubation of small tumor pieces with 10 ml of enzyme for 15 minutes at 37°C, followed by decanting the supernatant and reincubating the tumor with 10 ml of fresh enzyme for 10 minutes. In one instance, pronase digested tumor was washed 4 times with 25 ml of Dulbecco saline and incubated with 10 ml of collagenase (conc. 0.1 mg/ml) for 10 minutes. Mechanical preparation of the cells included a 15 minute stirring on ice or disruption of the tumor pieces in a syringe. As can be observed from Figure 41, enzyme treatment once again was found to remove surface bound TAIg. However, the collagenase treatment was found to retain most of the TAIg.

In order to study the effect of the type of enzyme treatment, the following enzymes were studied in the next experiment (conc. 0.1 mg/ml): trypsin, pronase and collagenase. The treatment included a half hour incubation of the excised and cultured cells with the respective enzymes at 37°C. The cell viability obtained with untreated

and trypsin, pronase and collagenase treatment was 20%, 63%, 71% and 85% respectively. All enzyme treatments retained most of the surface bound TAIg (Fig. 42). Binding of the RAM Ig to similarly treated cultured cells remained the same and the viability of the cells was not affected.

The above experiment was repeated with trypsin, pronase and collagenase, yielding viabilities of 65, 100 and 26% respectively. Once again the results showed a negligible loss of surface bound TAIg (Fig. 43).

In the sixth experiment, the effect of enzyme concentration on TAIg was studied. Pronase concentrations of 2.5, 1.25 0.5, 0.25 and 0.1 mg/ml were studied. As shown in Figure 44, loss of TAIg from the surface of CCH1 tumor cells was found to vary directly with enzyme concentration.

INFLUENCE OF REAGENT CONCENTRATION

The results of most experiments showed a direct correlation between RAM Ig concentration and its binding to cells. Three experiments which were particularly carried out to study the influence of reagent concentrations will be described in this section.

In the first experiment, reagent concentrations of 5, 1, 0.2 and 0.04 µg/100µl were studied and normal spleen and thymus cells were included in addition to CCH1 tumor cells. RAM Ig binding to the cells was found to vary directly with reagent concentration (Fig. 45). Moreover, at all reagent concentrations, highest binding was always observed on CCH1 tumor cells. Spleen cells exhibited lower RAM Ig binding whereas thymus cells showed only negligible binding.

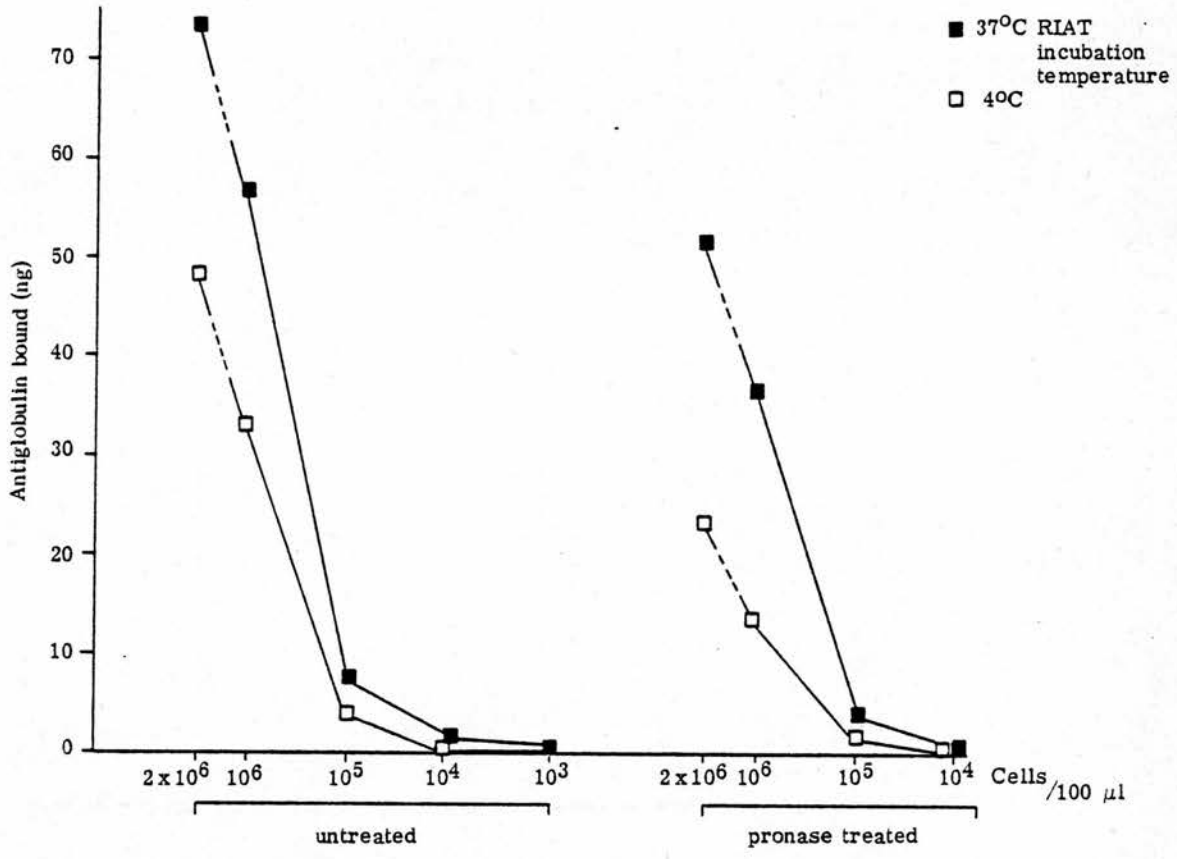


FIG 39 A COMPARISON BETWEEN MECHANICALLY AND ENZYME PREPARED TUMOR CELLS

Mechanical preparations consisted of mincing the tumor with scissors at 0-4°C. Enzyme preparation included 2 five minute incubations with pronase at 37°C. It can be observed that ^{125}I-RAM Ig binding to mechanically prepared tumor cells is usually higher than its binding to pronase prepared tumor cells. Note that reagent binding to cells varies positively with cell number and incubation temperature.

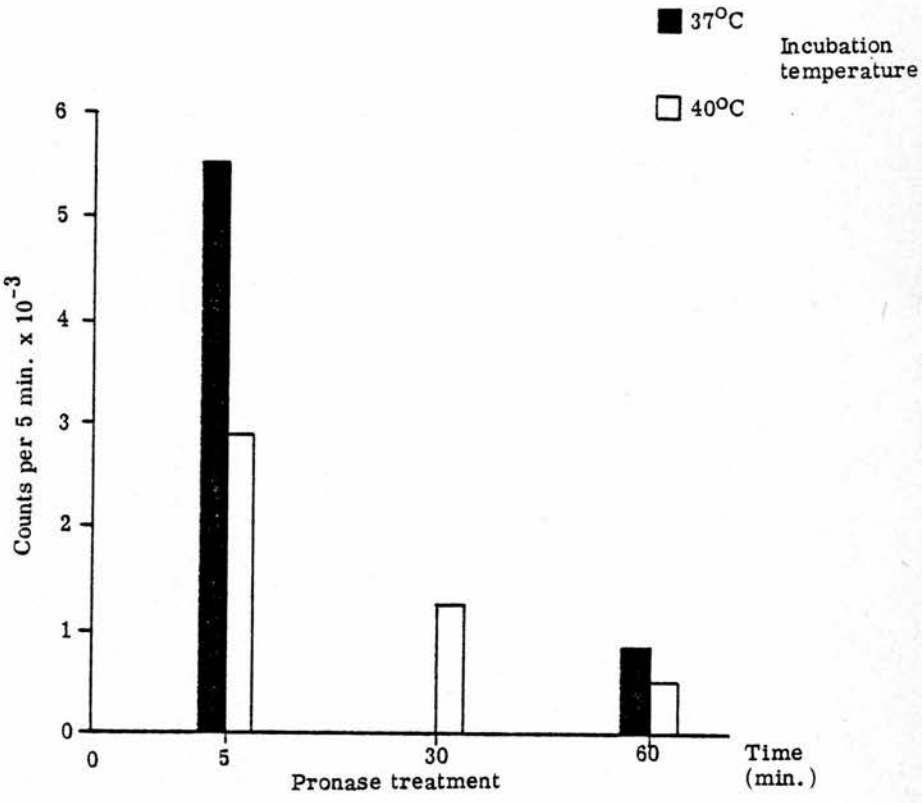


FIG 40 EFFECT OF ENZYME ON BOUND TUMOR ASSOCIATED IMMUNOGLOBULINS

The effect of pronase on tumor-associated immunoglobulins was studied in the direct radio-immune antiglobulin test.

Enzyme treatment consisted of incubating freshly excised tumor tissues with pronase for 5, 30 or 60 minutes. It can be observed that the loss of tumor-associated Igs from the surface of tumor cells increased with the enzyme incubation period. Note that ^{125}I-RAM Ig binding to cells varies positively with the incubation temperature used in the direct radio immune antiglobulin test.

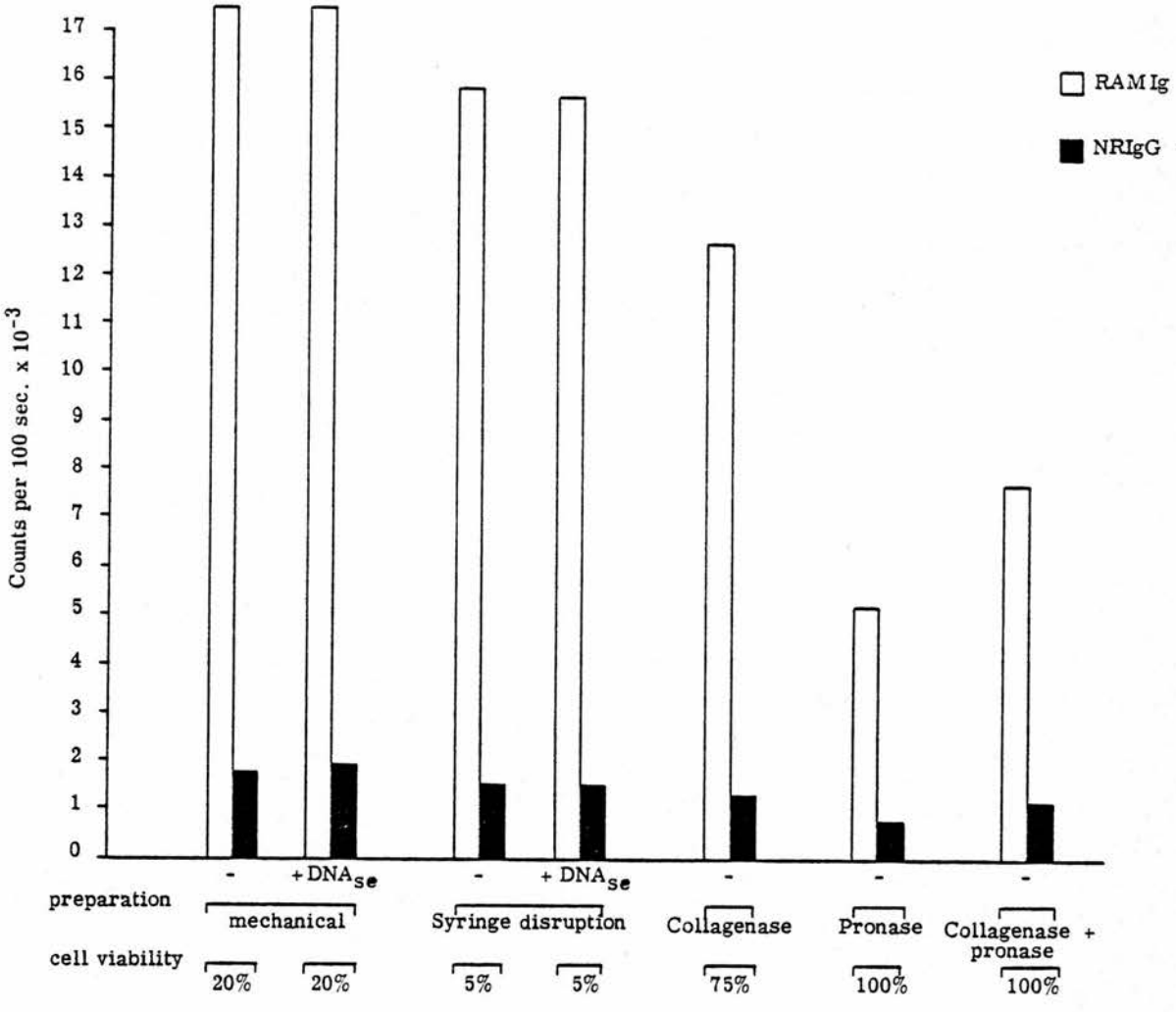


FIG 41 EFFECT OF ENZYME ON THE ^{125}I-RAM Ig BINDING TO TUMOR CELLS.

In this experiment the effect of DNAse, Collagenase and/or pronase on the RAM Ig binding to tumor cells was studied. The effect of DNAse was examined in order to determine whether the reagent was binding to surface-bound DNA liberated from dead cells. Moreover, collagenase (0.1 mg/ml) treated cells were compared with pronase (2.5 mg/ml) treated cells. It can be observed that a) DNAse treatment did not reduce the binding of RAM Ig to the cells; b) Pronase, whose concentration was higher than that of collagenase, removed surface bound tumor associated Igs. Note that the binding of NRIgG hardly exceeded background levels.

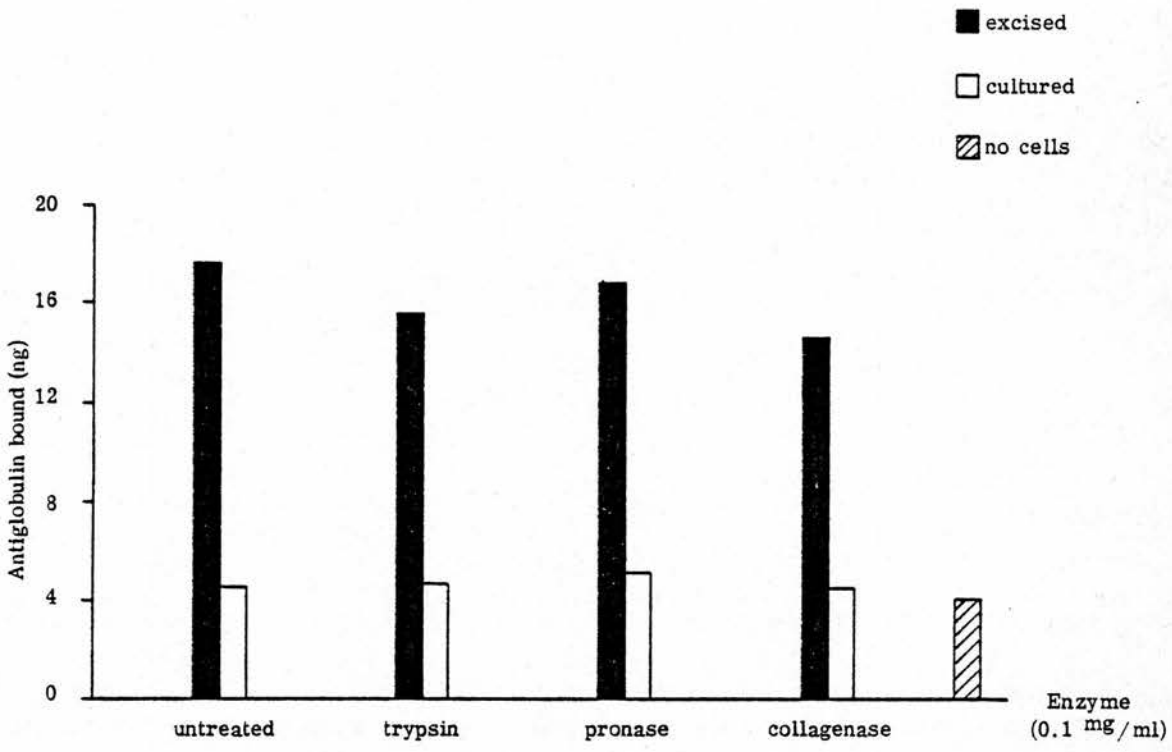


FIG 42 EFFECT OF ENZYME ON THE ^{125}I-RAM Ig BINDING TO TUMOR CELLS

Freshly excised and cultured CCH1 tumor cells with trypsin, pronase or collagenase (all at 0.1 mg/ml) were compared with nontreated tumor cells in the direct radio-immune antiglobulin test. Enzyme treatment included half an hour incubation of the tumor cells with the corresponding enzyme at 37°C. Control cells were incubated at 4°C for half an hour. It can be observed that surface bound tumor associated Igs were not effected by the low-concentrated enzymes. Note that reagent binding to cultured tumor cells is appreciably lower than its binding to freshly excised tumor cells.

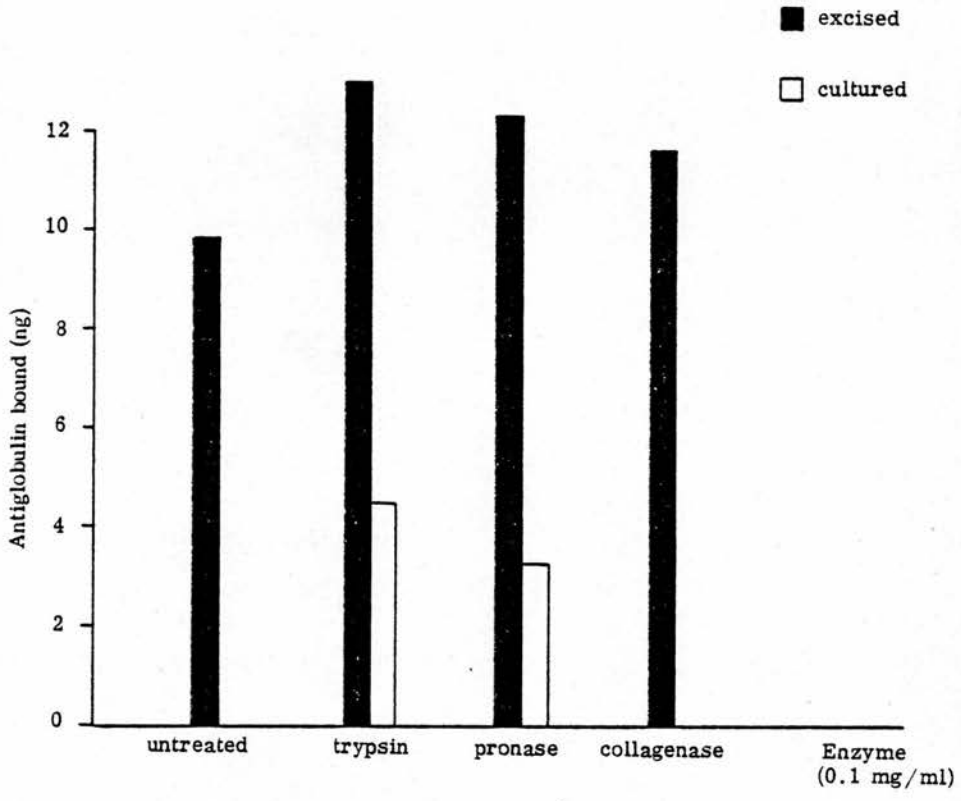


FIG 43 EFFECT OF ENZYME ON THE ^{125}I-RAM Ig BINDING TO TUMOR CELLS

Freshly excised and cultured CCH1 tumor cells treated with trypsin, pronase, or collagenase (all at 0.1 mg/ml) were compared with non-treated tumor cells in the direct radio-immune antiglobulin test. Enzyme treatment included half an hour incubation of the tumor cells with the corresponding enzyme at 37°C. Control cells were incubated at 4°C for half an hour. It can be observed that surface-bound tumor associated Igs were not effected by the low-concentrated enzymes. Note that reagent binding to cultured tumor cells did not exceed background levels.

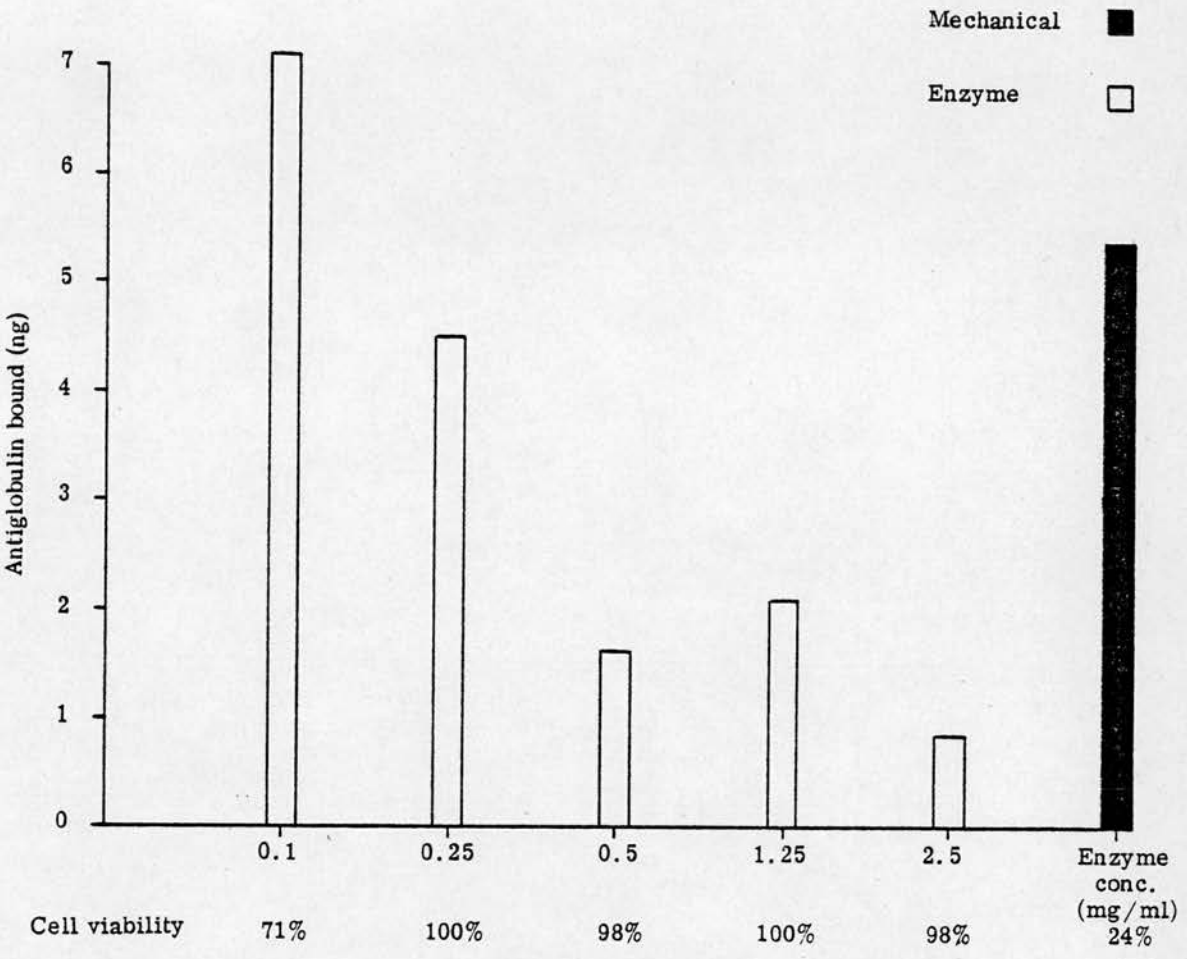


FIG 44 EFFECT OF ENZYME ON THE ^{125}I-RAM Ig BINDING TO TUMOR CELLS

In this experiment, the CCH1 tumor was disaggregated mechanically at 4°C or by incubation with various pronase concentrations in a 37°C water bath. The CCH1 cells were then washed and compared in the direct radio-immune antiglobulin test. Note that loss of TAIgs from the surface of the tumor cells varies positively with enzyme concentration.

RAM Ig concentrations of 1, 2 and 4 µg/100 µl were studied in the second experiment. Cells used included normal spleen cells, tissue culture CCH^1 cells and excised CCH^1 cells. As observed in Figure 46, reagent binding was found to vary directly with its concentration.

Finally, the effect of 2, 4 and 8 µg/100 µl concentrations of RAM Ig on normal spleen cells, CCH1 cultured and CCH1 excised tumor cells was studied. Reagent binding was once again found to be correlated with its concentration (Fig. 47).

For more examples on the influence of reagent concentration see Figures 37, 38, 48 and 51.

EFFECT OF INCUBATION TIME AND TEMPERATURE

The standard incubation period and temperature used in our assay was one hour at 4°C. However, to assess the effect of time and temperature on RAM Ig binding to tumor cells, different incubation periods at 4°C and 37°C were investigated.

Incubation periods of 1, 2, 3 and 4 hours were studied in the first experiment at temperatures of 4°C and 37°C. The RAM Ig concentrations used included 1, 0.5 and 0.125 µg/100 µl. As depicted in Figure 48, RAM Ig binding to the tumor cells increased with time and the reaction was enhanced at 37°C at the three reagent concentrations used. Moreover, as in previous experiments, the binding was found to vary directly with reagent concentration.

In the second experiment, incubation periods of 1.5, 3,5 and 20 hours were studied at 4°C and 37°C. Excised CCH^1 and T3 tumor cells and normal spleen cells were used. RAM Ig binding to the cells was once again found to vary directly with time (Fig. 49). However, the difference in the binding

observed between incubations at 4°C and 37°C was found to decrease and, in the case of T3 and spleen cells, was reversed at 20 hours. Excised CCH1 cells exhibited highest binding of the RAM Ig followed by T3 and spleen cells.

In the third and final experiment, incubation periods of ½, 1, 2 and 20 hours at 4°C were studied. Similar to the previous results, RAM Ig binding to the cells was found to increase with time (Fig. 50).

For more examples of the effect of incubation temperature on RAM Ig binding see Figures 37, 39 and 40.

EFFECT OF CELL CONCENTRATION

Most of the experiments carried out for the development of the direct RIAT included a study of the effect of cell concentration on RAM Ig binding. In all the experiments, reagent binding was found to vary directly with cell concentration (see Figures 36, 37, 38, 39, 45, 51 and 52). With the exception of Figure 51, all the figures represent a study of cell concentration in conjunction with other parameters the details of which are described in the respective sections. Figure 51 represents a study in which RAM Ig binding to 5×10^5, 5×10^6 and 1×10^7 cells/ml was compared. The cells consisted of excised CCH1 tumor cells, cultured CCH1 tumor cells and normal spleen cells. A direct relationship between cell concentration and RAM Ig was again indicated. Moreover, the difference between RAM Ig binding to excised CCH1 cells and spleen cells was amplified at higher cell concentrations probably due to the larger size of tumor cells.

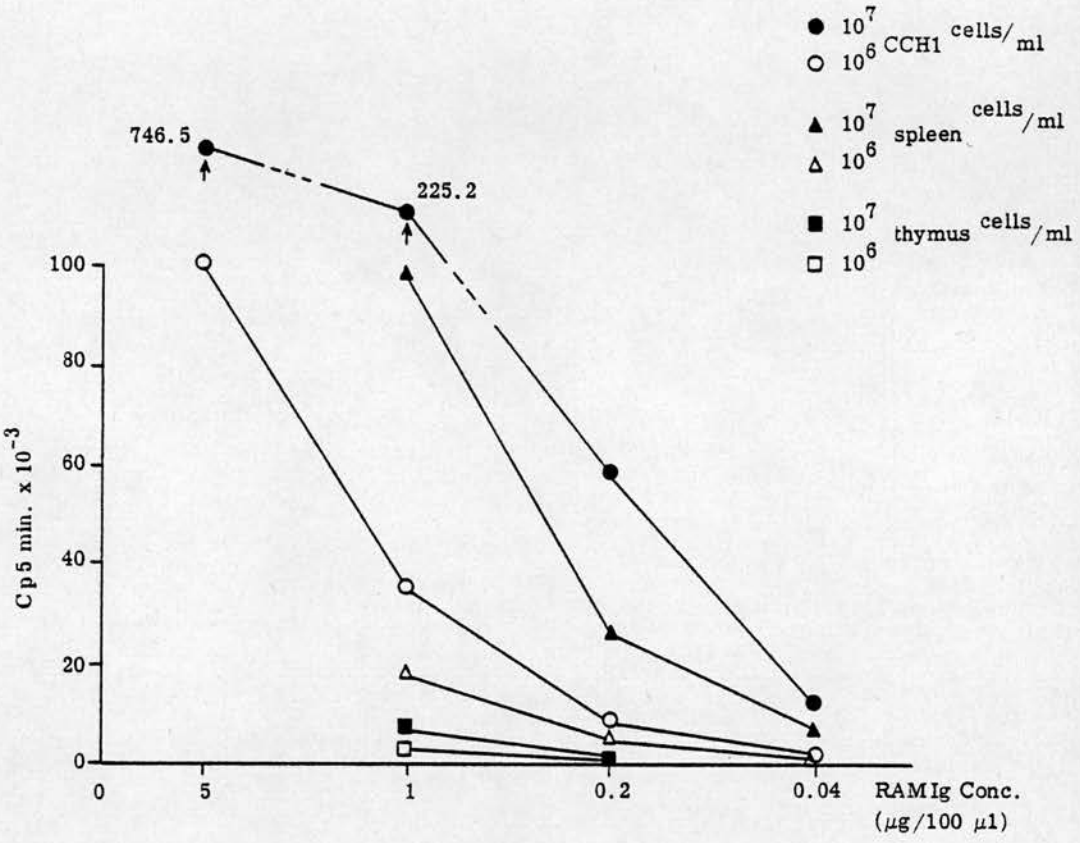


FIG 45 DETERMINATION OF THE OPTIMUM ^{125}I-RAM Ig CONCENTRATION IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST.

In this experiment, the effect of RAM Ig concentration on its binding to cells was examined at 2 different numbers. Cells studied included freshly excised CCH1 tumor cells, spleen cells, and thymus cells. It can be observed that the binding of RAM Ig to the cells varies positively with its concentration. Note that a) RAM Ig binding is highest in CCH1 tumor cells; b) reagent binding increases with the number of cells.

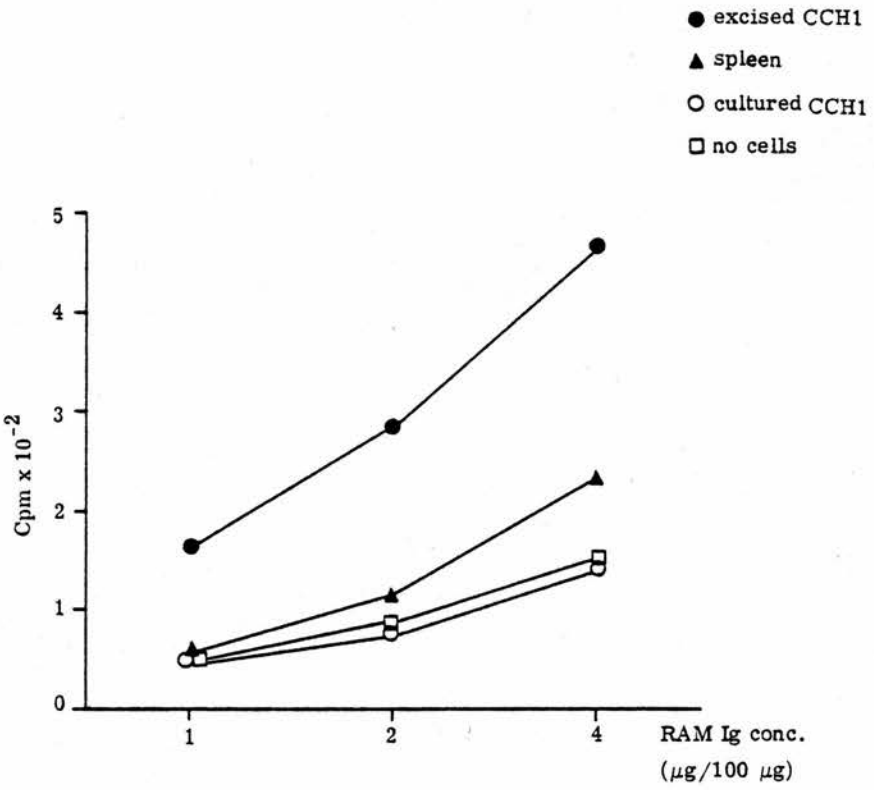


FIG 46 DETERMINATION OF THE OPTIMUM ^{125}I-RAM Ig CONCENTRATION IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST.

In this experiment, the effect of RAM Ig concentration on its binding to cells was examined. Cells studied included freshly excised and cultured CCH1 tumor cells, and spleen cells. It can be observed that RAM Ig binding to the cells varies positively with its concentration. Note that a) RAM Ig binding is highest in freshly excised CCH1 cells; b) reagent binding to cultured tumor cells does not exceed background levels.

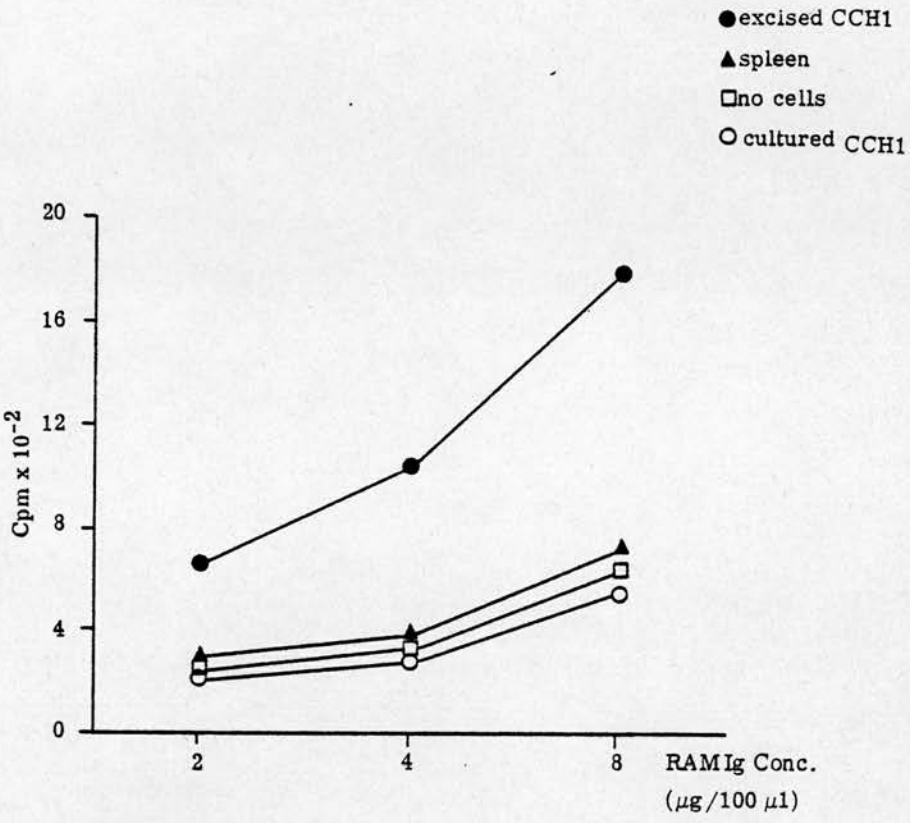


FIG 47 DETERMINATION OF THE OPTIMUM ^{125}I-RAM Ig CONCENTRATION IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST.

In this experiment, the effect of RAM Ig concentration on its binding to cells was examined. Cells studied included freshly excised and cultured CCH_1 tumor cells, and spleen cells. It can be observed that RAM Ig binding to the cells varies positively with its concentration. Note that a) RAM Ig binding is highest in freshly excised CCH^1 cells; b) reagent binding to cultured tumor cells does not exceed background levels

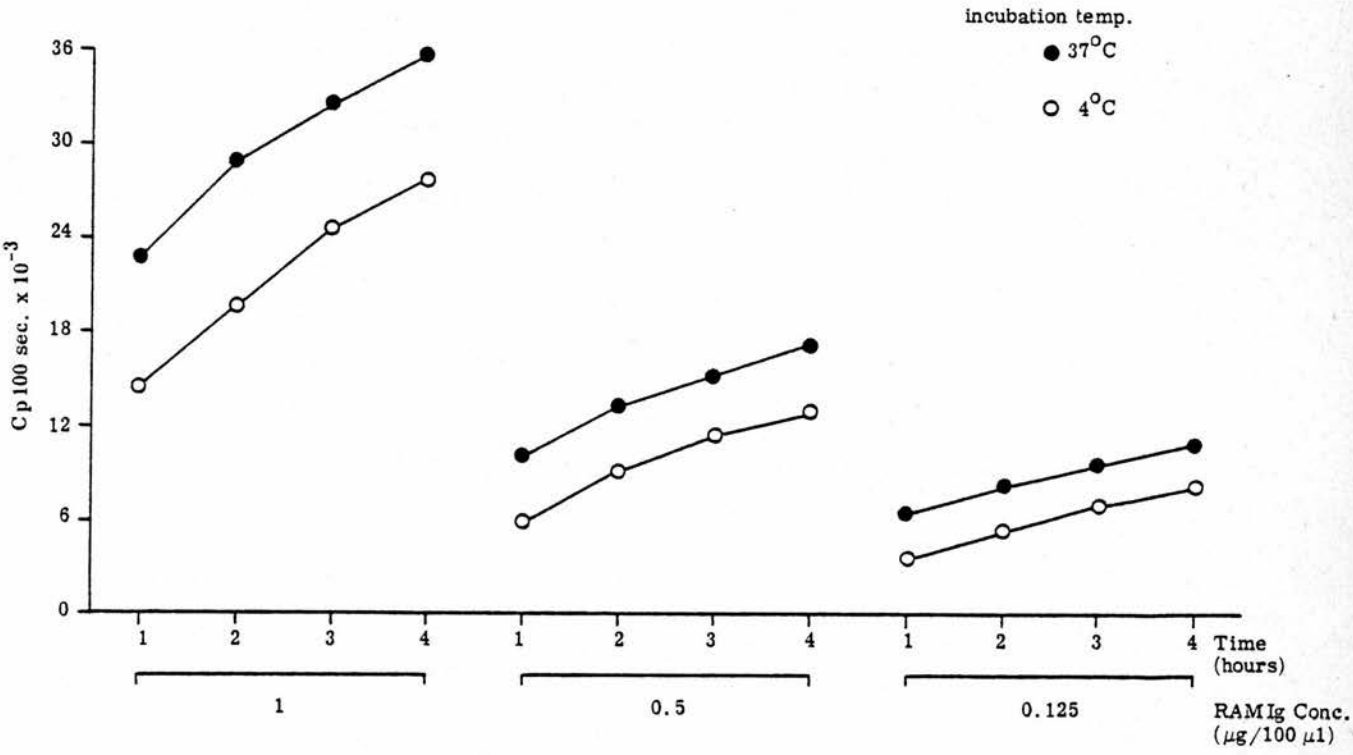


FIG 48 DETERMINATION OF THE OPTIMUM INCUBATION TIME IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST

In this experiment, the effect of incubation time on reagent binding to freshly excised CCH1 tumor cells was examined at 2 different incubation temperatures. Three different reagent concentrations were used. It can be observed that at both incubation temperatures, RAM Ig binding to tumor cells gradually increased with incubation time. Note that reagent binding to the cells varies positively with its concentration.

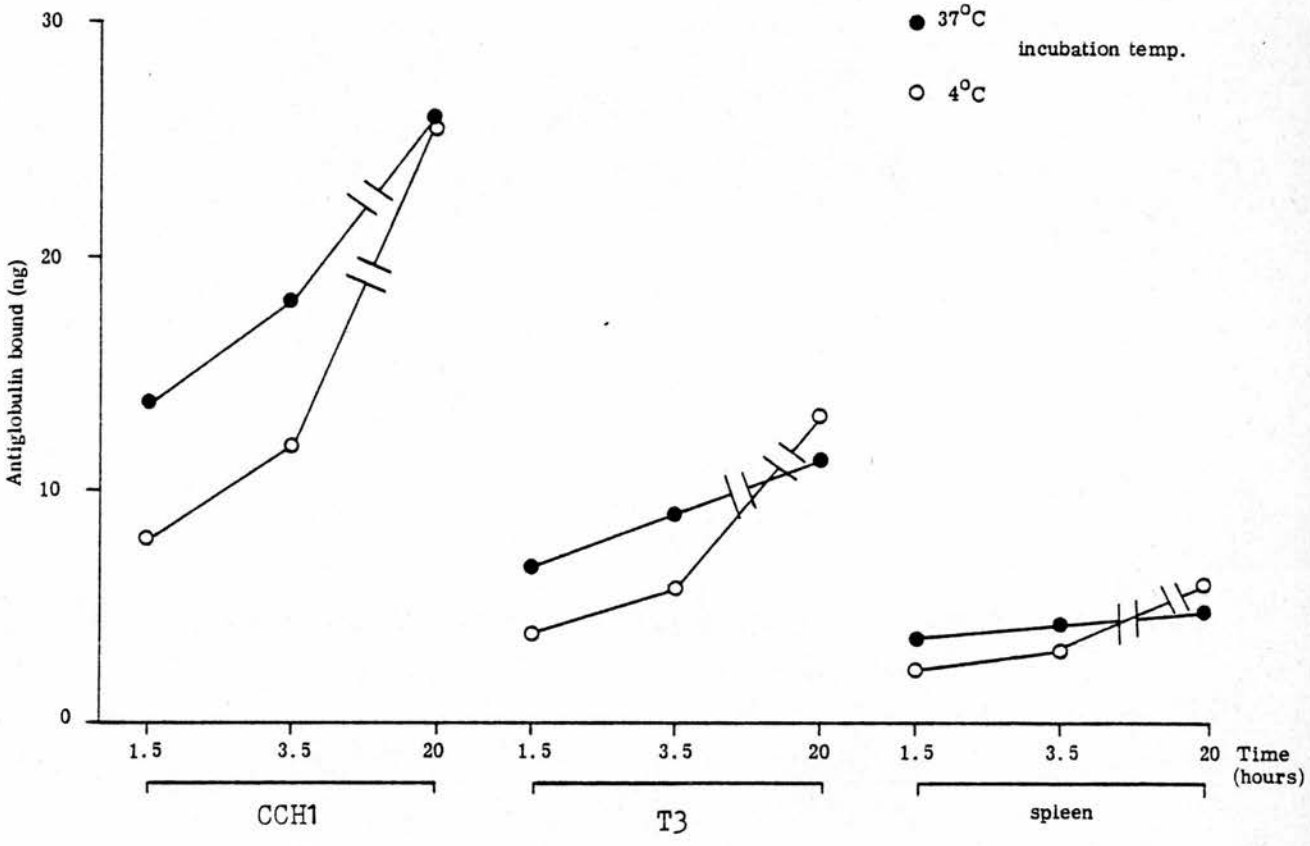


FIG 49 DETERMINATION OF THE OPTIMUM INCUBATION TIME IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST.

In this experiment, the effect of incubation time on reagent binding to freshly excised CCH1, T3, and spleen cells was examined at 2 different incubation temperatures. It can be observed that at both incubation temperatures, RAM Ig binding to the cells increased with incubation time. Note that a)RAM Ig binding is highest in CCH1 tumor cells; b)the differences observed at the 2 incubation temperature minimize as the incubation time increases.

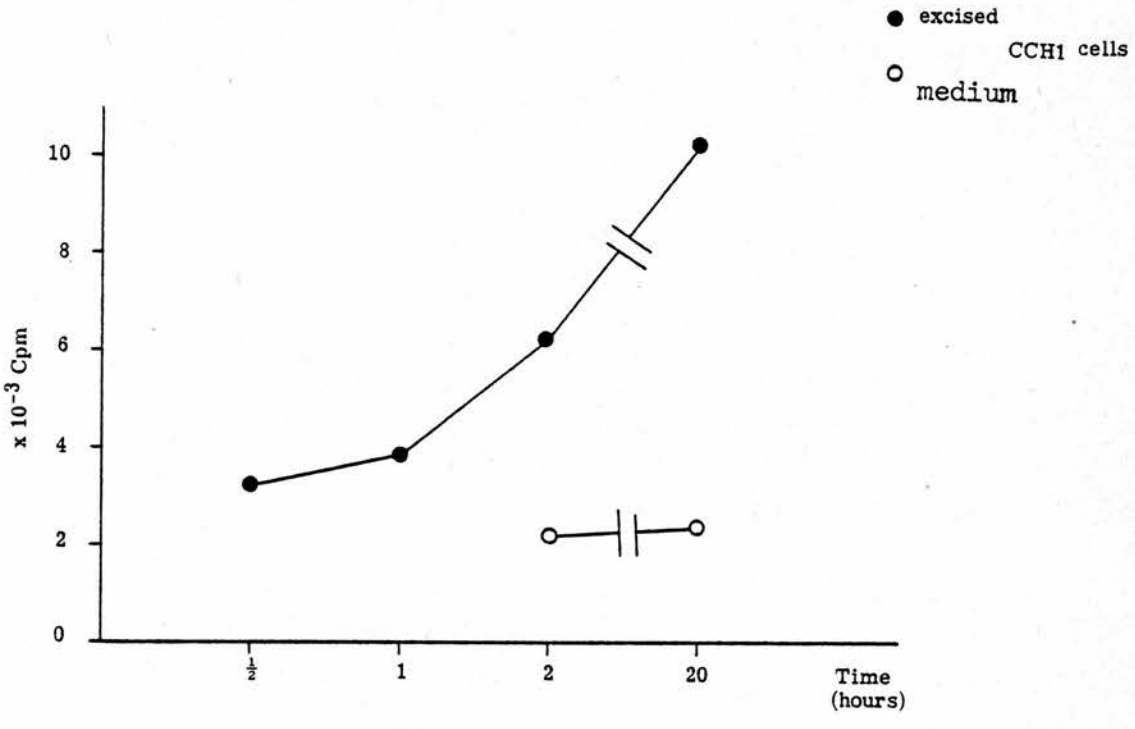


FIG 50 DETERMINATION OF THE OPTIMUM INCUBATION TIME IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST

In this experiment, the effect of incubation time on reagent binding to freshly excised CCH1 tumor cells was examined. It can be observed that RAM Ig binding to the tumor cells gradually increases with incubation time.

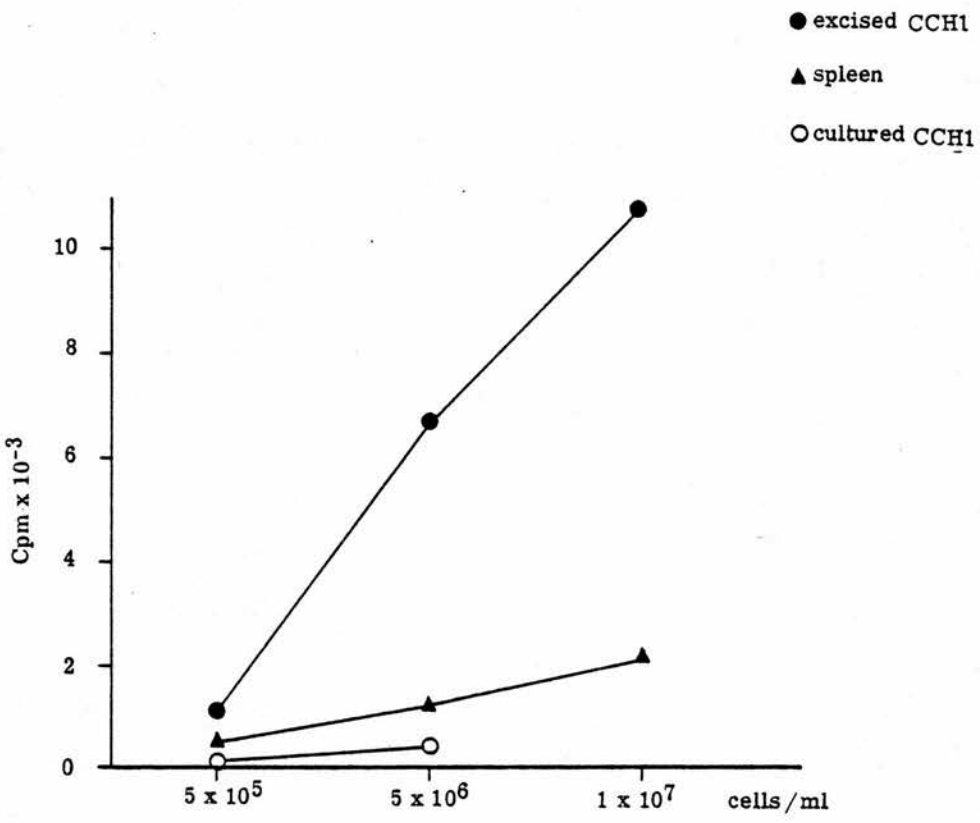


FIG 51 DETERMINATION OF THE OPTIMUM CELL NUMBER IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST

In this experiment, the effect of cell number on reagent binding to freshly excised and cultured CCH1 tumor cells, and spleen cells was studied. It can be observed that RAM Ig binding to the cells varies positively with cell number. Note that RAM Ig binding is highest in freshly excised CCH1 cells.

ASSESSMENT OF NRIgG BINDING

Some of the RAM Ig nonspecific binding observed to tumor cells could have been due to NRS. In order to find out whether NRIgG binds to tumor cells, immune and nonimmune RIgG were compared. Immune RIgG comprised an acid eluted RAM Ig (see Materials and Methods) and nonimmune RIgG included a NH_4SO_4 precipitate of NRS consecutively passed through a DEAE column. Reagent concentrations of 1000, 200, 40 and 8 ng/0.1 ml were used, and as can be observed from Figure 52, immune RAM Ig exhibited a much higher binding to the tumor cells than NRIgG.

In another experiment, immune RAM Ig and NRIgG were compared on mechanically and enzyme prepared cells, where again the former was found to bind much more excessively to the cells (see Figure 41).

NONSPECIFIC BINDING TO DEAD CELLS

One experiment was carried out in order to determine the RAM Ig binding to dead cells. Non treated CCH1 cultured cells (viability 100%) were compared with CCH1 cultured cells incubated at 48°C for 2 hours (viability 30%). No significant difference was observed between dead and live cells in their RAM Ig binding (Figure 53).

A STUDY OF $F(ab')_2$ BINDING

In order to determine whether normal immunoglobulins bind to the tumor cells through their Fc-portion, binding of NRIgG and its $F(ab')_2$

derivative to the cells was compared. The $F(ab')_2$ fraction was prepared by pepsin digestion of the NRIgG followed by the recovering of the $F(ab')_2$ on G150 sephadex column. Both reagents were absorbed with CCH1 tumor culture cells and adjusted to the same concentration. As observed in Figure 54, binding of the NRIgG to the excised CCH1 cells was found to decrease upon cleavage of its Fc portion, appreciably so at 10^7 cells/ml but less significantly at 10^6 and 10^5 cells/ml. Since our RIAT assays employ 10^5 cells/ml, the RAM Ig binding to cells through its Fc portion may be insignificant.

SUMMARY

The optimal conditions for the direct RIAT were found to be almost identical to those of the indirect RIAT. The RAM Ig binding to the tumor cells proved to be directly correlated with reagent concentration, incubation time and temperature and cell number.

Precoating studies showed 5% FCS to be a suitable inhibitor of non specific binding of the RAM Ig to the plastic.

Enzyme studies indicated that minute amounts of trypsin, pronase or collagenase were sufficient to yield highly viable cells without removing the surface bound TAIg.

RAM Ig binding to the tumor cells was found to be specific: binding was restricted to excised tumor cells, normal spleen cells and cultured tumor cells treated with serum from tumor-bearing mice; normal thymus cells and cultured tumor cells did not bind the RAM Ig; immune RAM IgG binding to tumor cells appreciably exceeded that of nonimmune NRIgG; and RAM Ig did not bind more to dead cultured tumor cells as compared to live cells.

Finally, immunoglobulin binding to tumor cells via their Fc portion was found to be insignificant at the cell number used in our assay.

The following conditions were adopted for the application of the direct RIAT :

a. an overnight precoating of the tubes at $4^{\circ}C$ with 4 ml of 5% FCS PBS per tube
b. a cell number of $5 \times 10^4/100\ \mu l$
c. a RAM Ig concentration of $4\ \mu g/100\ \mu l$
d. an incubation period and temperature of 1 hour and $4^{\circ}C$ respectively (or on ice)
e. washing the cells with 5% FCS PBS

A temperature of $4^{\circ}C$ instead of $37^{\circ}C$ was chosen for the incubation period in order to maintain the conditions used in the indirect RIAT.

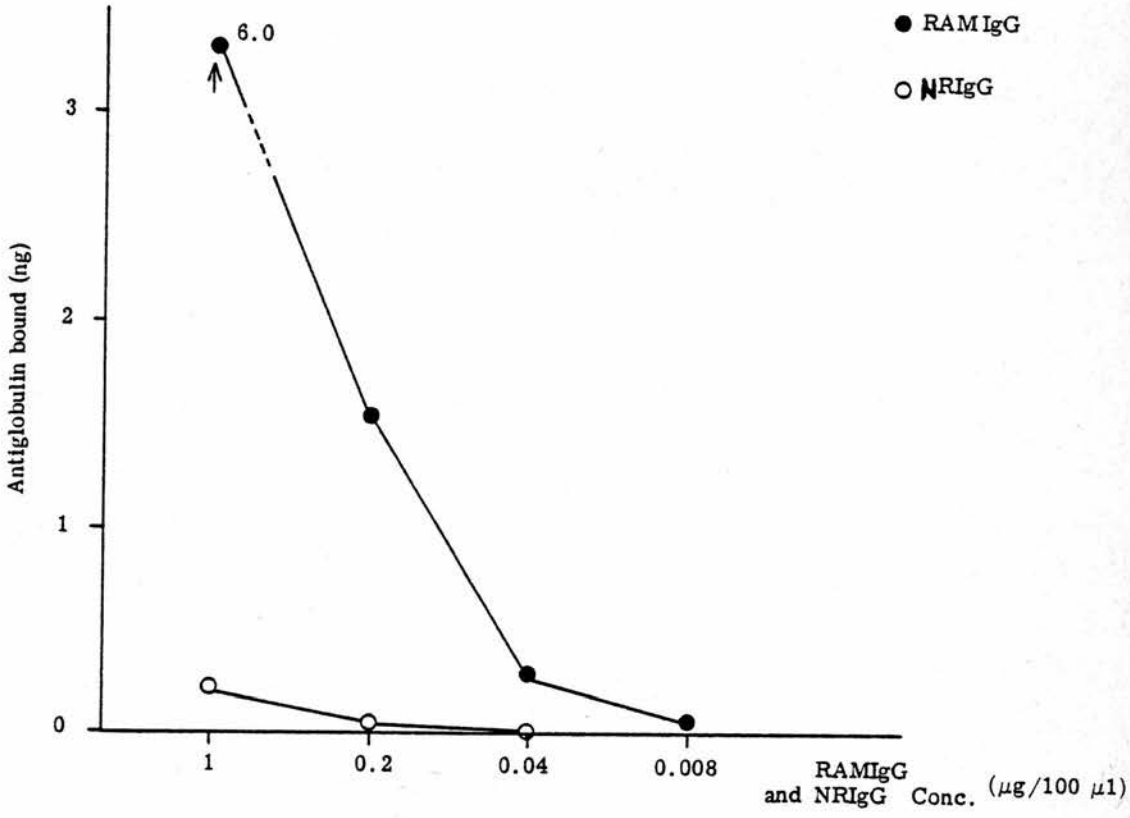


FIG 52 NONSPECIFIC BINDING OF NRIgG TO TUMOR CELLS IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST.

In this experiment, the nonspecific binding of NRIgG to freshly excised CCH1 tumor cells was assessed at various reagent concentrations. It can be observed that at all the reagent concentrations used, RAM Ig binding to tumor cells appreciably exceeded that of NRIgG binding. Note that RAM Ig binding to the cells varies positively with its concentration.

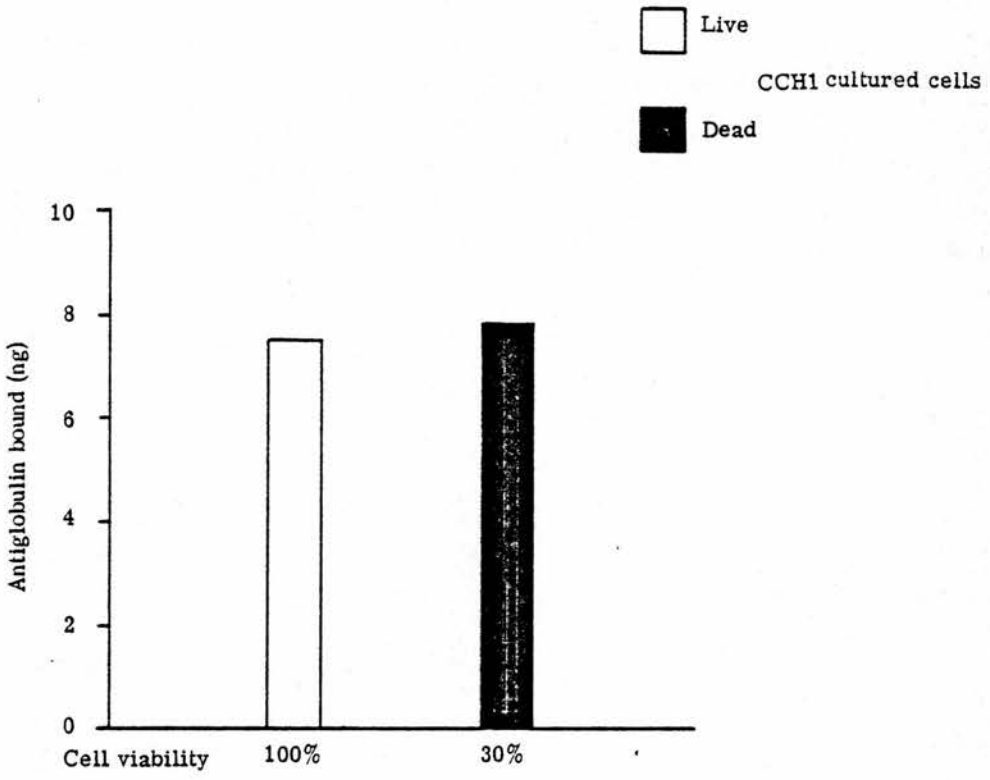


FIG 53 THE EFFECT OF DEAD CELLS ON REAGENT BINDING IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST.

In this experiment, cultured CCH1 tumor cells were divided into 2 tubes, one of which was incubated in a 48°C water bath for 2 hours, while the other was left at 4°C for 2 hrs. The viability of the cells incubated at 48°C dropped from 100% to 30%. The live and dead cells were compared in the direct radio immune antiglobulin test. Note that there is no difference in RAM Ig binding between live and dead cells.

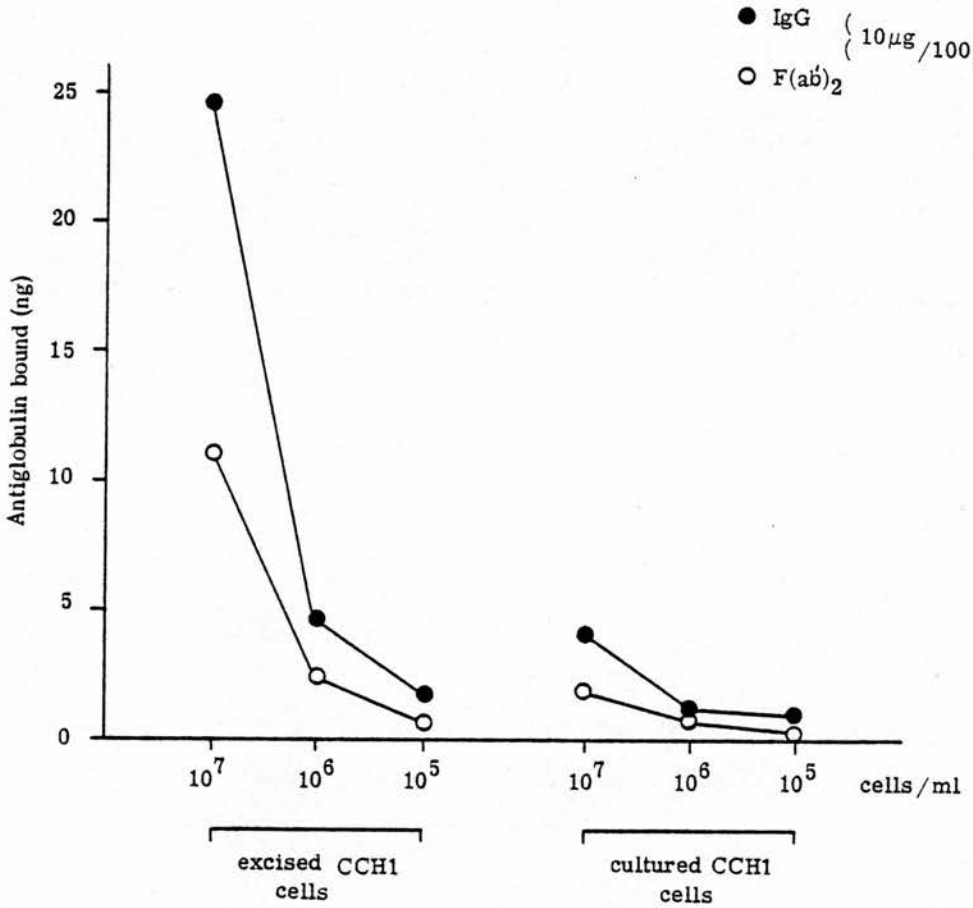


FIG 54 ASSESSMENT OF REAGENT BINDING TO TUMOR CELLS THROUGH THEIR FC-PORTION IN THE DIRECT RADIO IMMUNE ANTIGLOBULIN TEST.

In this experiment, the binding of NRIgG to freshly excised and cultured CCH1 tumor cells was compared with the binding of its $F(ab')_2$ derivative at various cell numbers. It can be observed that NRIgG binds more to the freshly excised tumor cells than its $F(ab')_2$ derivative. However, the observed difference decreases at lower cell numbers. Note that a similar difference in binding is not observed in cultured tumor cells. While this result may suggest that reagents could nonspecifically bind to infiltrating host cells via their Fc-portion, such binding would be insignificant at the cell number used in the standard direct radio immune anti globulin test (i.e. 5×10^5 cells/ml).

APPLICATION OF THE DIRECT RIAT

The direct radioimmune antiglobulin test (RIAT) was used to repeat experiments carried out by the indirect RIAT and to perform additional experiments. Consistent results were obtained regarding the *in vivo* binding of immunoglobulins to T3 and CCH1 tumor cells, to tumors produced by excised and cultured tumor cells, and to tumors in *C.parvum* treated and nontreated mice. The TAIg levels were again found to be dose dependent and the amount tended to vary directly with size and age of tumor. Morover, in order to determine whether the results in different mice could be reproduced in the same mouse, comparative studies were also carried out on opposite limbs of the same mouse.

THE RESPONSE TO CCH1 AND T3 TUMORS IN DIFFERENT MICE

An experiment was carried out in which the variation of *in vivo* bound TAIg with age and size of CCH1 and T3 tumors grown in different mice was simultaneously studied. As can be observed in Figures 55 and 56 the *in vivo* bound TAIg was found to increase with the age and size of both types of tumors. Moreover, CCH 1 tumor cells always exhibited higher amounts of TAIg than T3 tumor cells. The reasons behind the decrease in TAIg on day 18 have not been elucidated but its implications are discussed later (see Discussion).

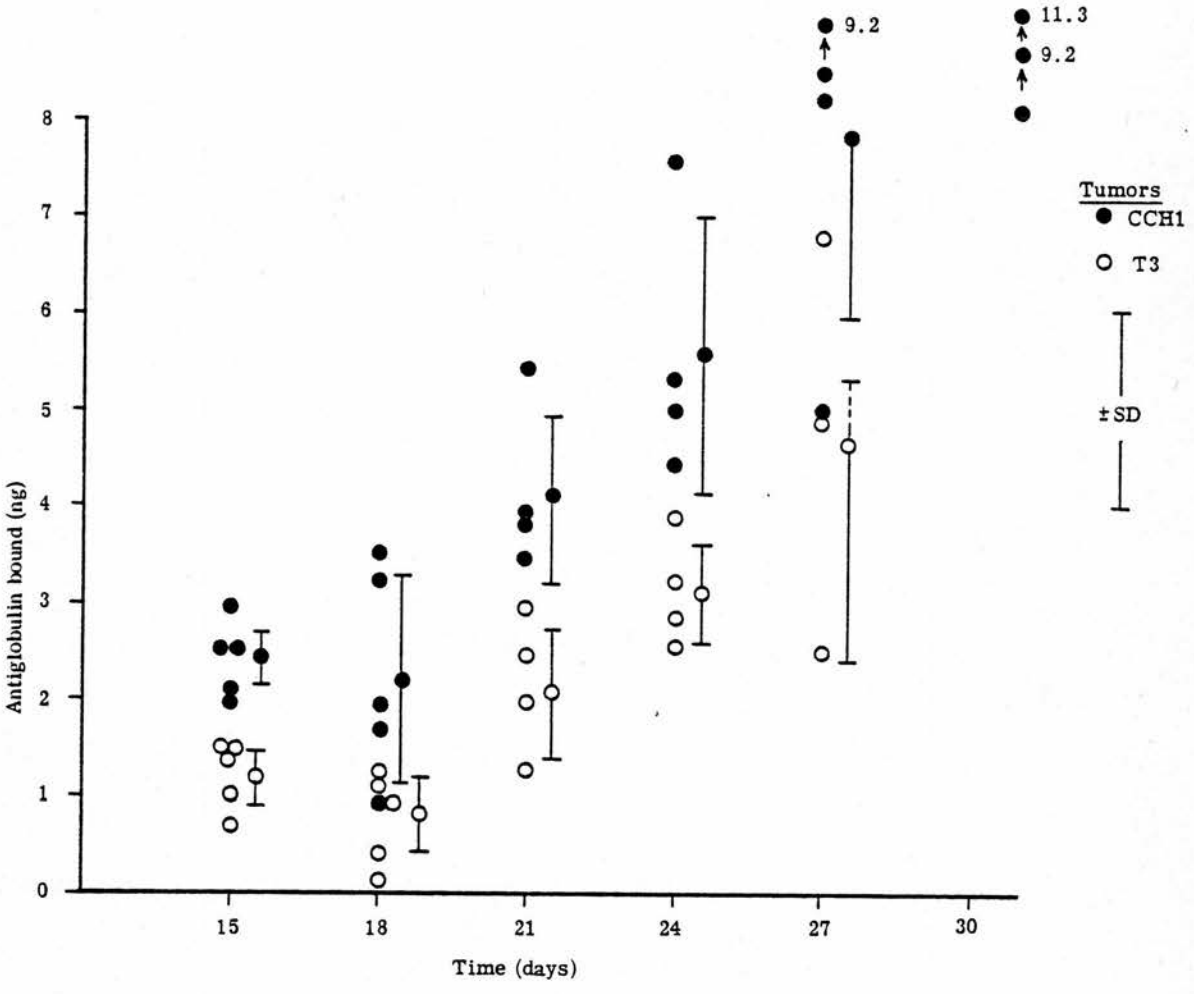


FIG 55 THE VARIATION OF TAIg BINDING WITH TIME IN CCH1 AND T3 TUMORS

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 or T3 tumor cells. At various times thereafter, the amount of host Ig associated with the surface of 5 x 10^4 cells from individual tumors was assessed by the direct radio immune antiglobulin test. It can be observed that TAIg binding increases with time in both CCH1 and T3 tumors. Note that TAIg is consistently less on T3 tumor cells than CCH1 cells.

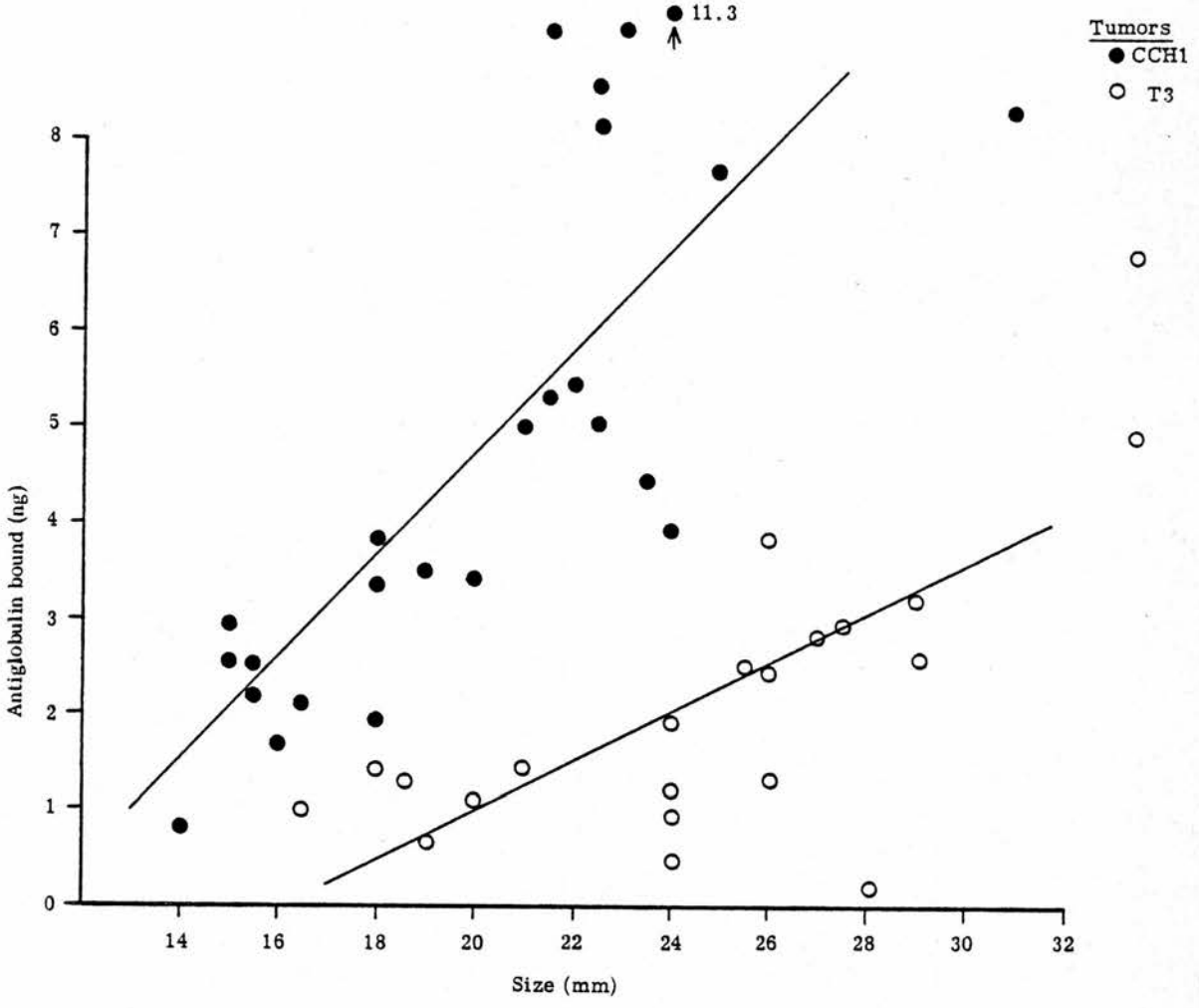


FIG 56 THE VARIATION OF TAIg BINDING WITH TUMOR SIZE IN CCH1 AND T3 TUMORS

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 or T3 tumor cells. The figure depicts tumor size and the amount of host Ig associated with the surface of 5 x 10^4 cells from the corresponding tumor in the experiment carried out on CCH1 and T3 tumor bearing mice (see Figure 55). It can be observed that there is a positive correlation between TAIg binding and tumor size in both CCH1 and T3 tumors (For a similar assessment of TAIg binding with CCH1 tumor size in the indirect RIAT, refer to Figure 26).

THE RESPONSE TO CCH1 TUMORS IN C.PARVUM TREATED AND NONTREATED MICE

The effect of C. parvum on tumor growth and in vivo bound TAIg was studied. C. parvum treatment included an ip injection of 0.2 ml (1.4 mg) C. parvum on the third day following the injection of tumor cells. As observed with the indirect RIAT, tumor growth inhibition in C. parvum treated mice was again found to be correlated with higher in vivo bound TAIg in the tumors (Figures 57 and 58). Moreover, in treated and nontreated mice, in vivo bound TAIg increased with the age of the tumors and, in nontreated mice only, with the size of the tumors. However, as in the previous experiment, a decrease in TAIg was noted on day 18.

THE RESPONSE TO EXCISED AND CULTURED CCH1 TUMOR CELLS

The response to excised and cultured CCH[1] tumors was studied in one experiment. Unlike the other experiments in which tumor cells were prepared mechanically, the tumor cells in this experiment were prepared by incubating tumor tissues in 5 mls pronase (0.1 mg/ml) at 37°C for ½ an hour. Tumors grown from freshly excised tumor cells were found to bind higher TAIg in vivo than those grown from cultured tumor cells (Fig. 59).

THE RESPONSE TO CCH1 INJECTED INTO OPPOSITE LIMBS IN THE SAME MOUSE

In order to determine whether the effects observed in different mice could be reproduced in the same mouse, tumors grown on opposite limbs of

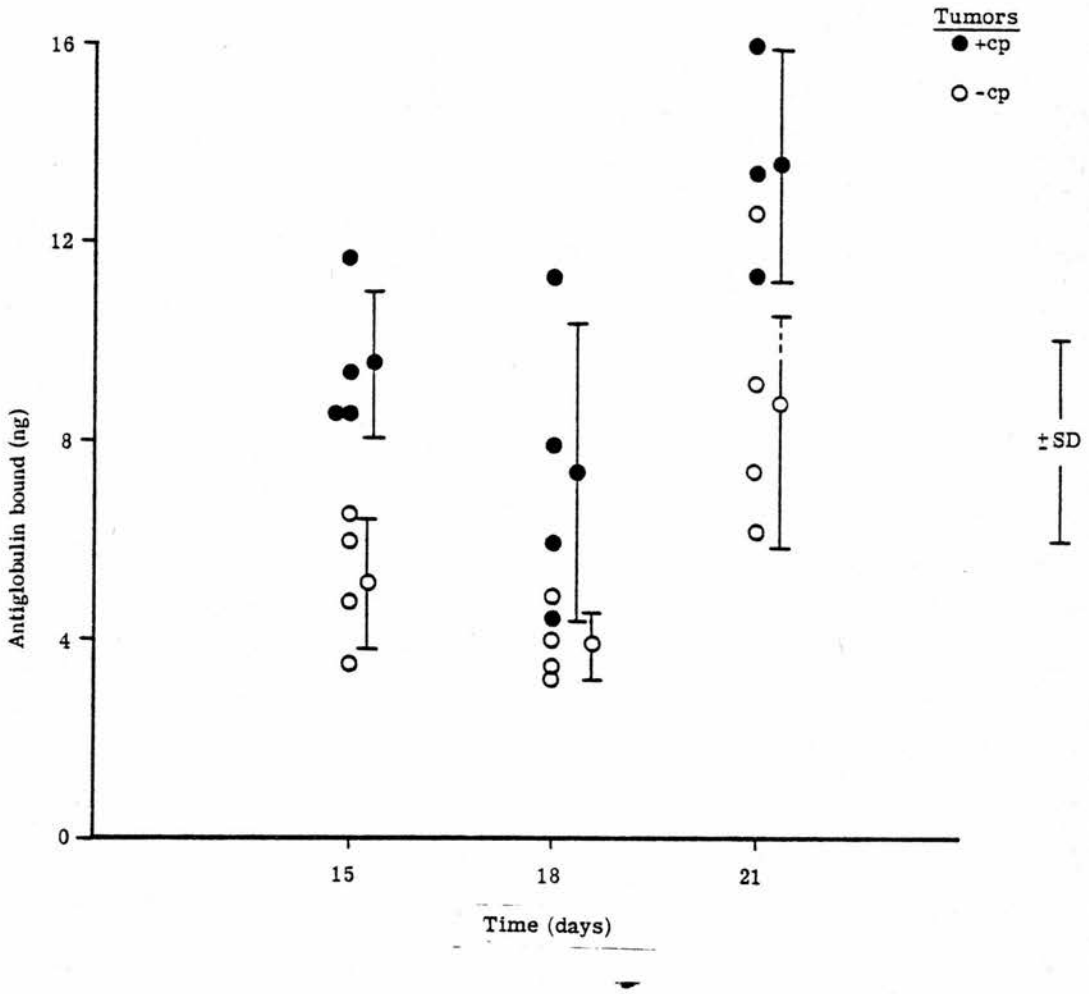


FIG 57 THE VARIATION OF TAIg BINDING WITH TIME IN C.PARVUM TREATED AND NONTREATED CCH1 TUMOR BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 tumor cells. On Day 3, half of the mice were injected ip with 1.4 mg of C.parvum. At various times thereafter, the amount of host Ig associated with the surface of 5 x 10^4 cells from individual tumors was assessed by the direct radio immune antiglobulin test. It can be observed that TAIg binding increases with time in both C.parvum treated and nontreated tumor bearing mice. Note that C.parvum treated mice exhibit higher TAIg than nontreated mice at all the times tested.

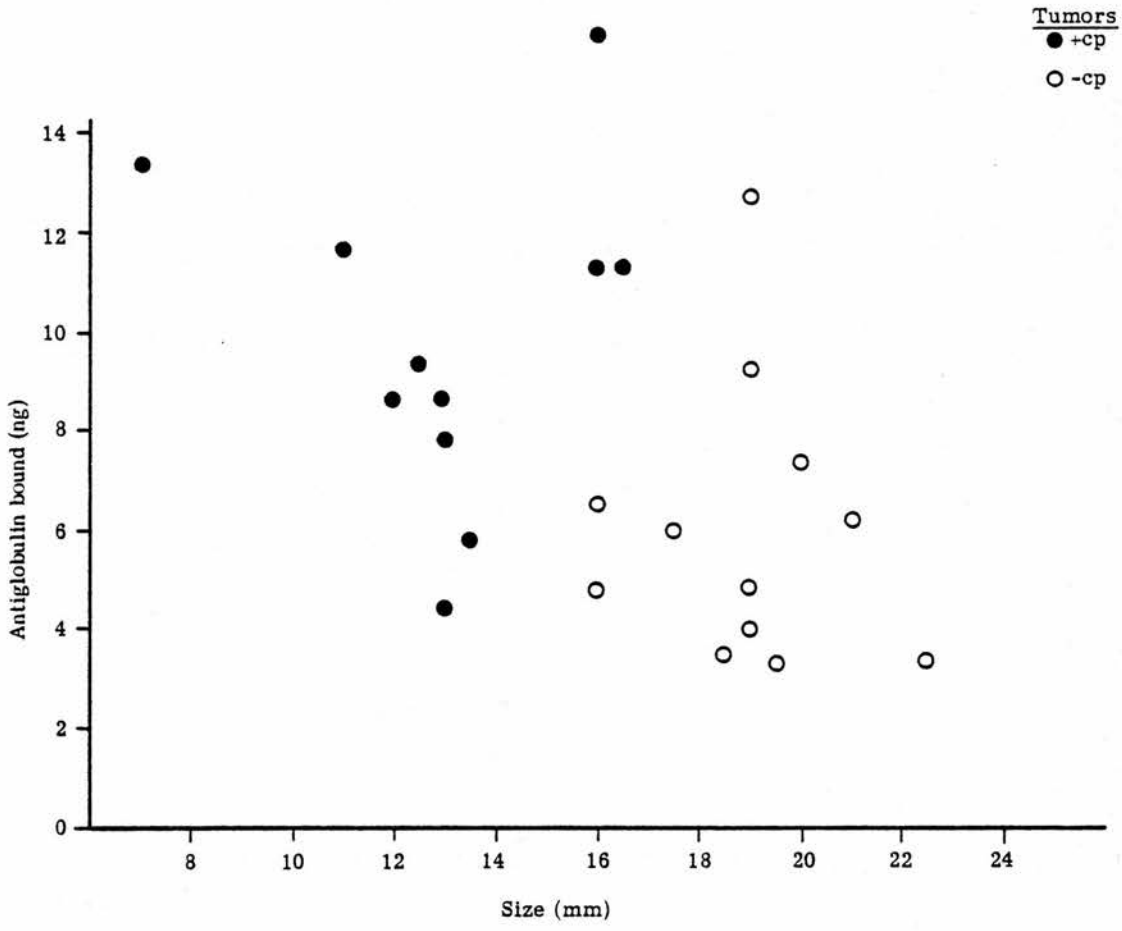


FIG 58 THE RELATIONSHIP OF TAIg BINDING TO TUMOR SIZE IN C.PARVUM TREATED AND NONTREATED CCH1 TUMOR BEARING MICE

CBA mice were injected s.c. on Day 0 with 10^6 viable CCH1 tumor cells. On day 3, half of the mice were injected ip with 1.4 mg of C.parvum. The figure depicts tumor size and the amount of host Ig associated with the surface of 5×10^4 cells from the corresponding tumor in the experiment carried out on C.parvum treated and non-treated tumor bearing mice (see Figure 57). While the number of tumors is too small for significant observations on the relationship of TAIg with tumor size, it can be noted that the smaller tumors from C.parvum treated mice have higher TAIg than the larger tumors from nontreated mice.

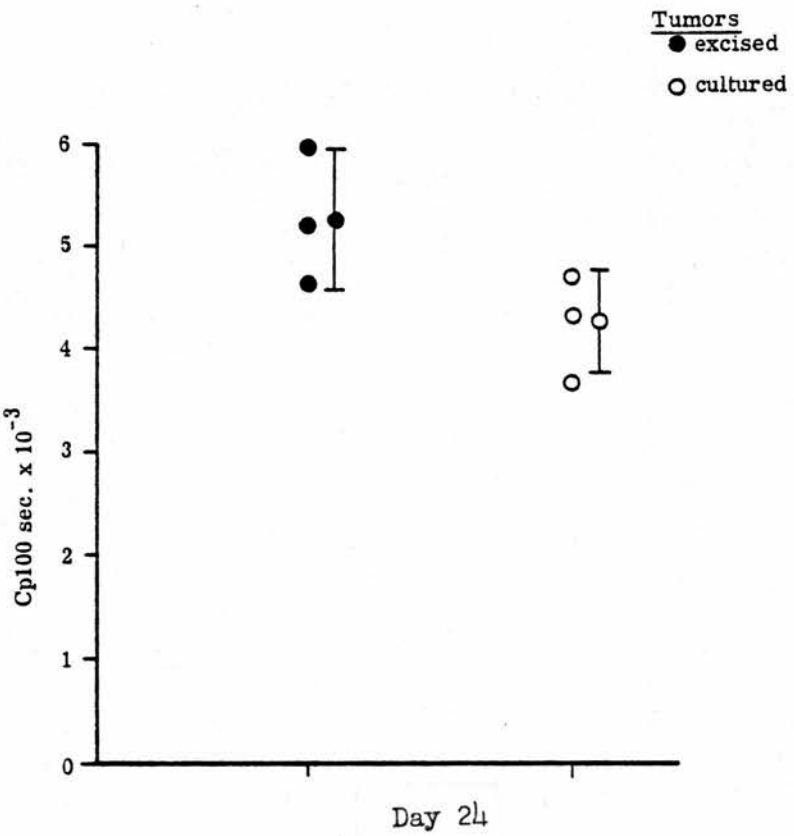


FIG 59 THE RESPONSE TO FRESHLY EXCISED AND CULTURED CCH1 TUMOR CELLS

CBA mice were injected s.c. on Day 0 with 10^6 viable freshly excised or cultured tumor cells. On day 24, the amount of host Ig associated with 5 x 10^4 cells from the individual tumors was assessed by the direct radio immune antiglobulin test. Note that tumors grown from freshly excised tumor cells exhibit higher TAIg than tumors grown from cultured tumor cells.

mice were studied in 2 experiments.

Prior to comparative studies between CCH1 and other types of tumors in the same mouse, CCH1 tumors grown on opposite limbs were compared in order to insure that in vivo TAIg binding was equal on both limbs. 1×10^6 CCH1 cells were subcutaneously injected into each hind limb of 10 mice. Five mice were sacrificed on days 16 and 20 by ether inhdation, and their tumors excised and studied. On both days, no significant difference in in vivo bound TAIg or size between tumors grown on opposite limbs of the mice was observed (Figs. 60 and 61). A slight increase of in vivo bound TAIg with time was noted on all the tumors studied. Similar results were obtained with the other experiment.

THE RESPONSE TO CCH1 AND T3 TUMORS IN THE SAME MOUSE

In vivo bound TAIg in CCH1 and T3 tumors grown on opposite limbs of the same mouse was compared in three experiments. CCH1 tumor cells consistently showed higher in vivo bound TAIg than T3 tumor cells. Although the tumors studied were approximately of the same age (15-17 days), the mean TAIg amounts bound varied between the experiments owing to the different ^{125}I-RAM Ig reagents used in each experiment.

In the first experiment, 17 day old CCH1 and T3 tumors were studied. CCH1 tumors showed a mean TAIg amount of 3.2 ng and a mean size of 18.6 mm compared to 0.98 ng and 22.7 mm respectively in T3 tumors (Fig. 62a).

In the second experiment, 16 day old tumors were compared. CCH1 tumors once again showed a higher mean TAIg amount of 11.6 ng relative to 2.4 ng in T3 tumors (Fig. 62b).

Day 15 tumors were studied in the third experiment. Similar to the previous two experiments, CCH1 tumors exhibited higher TAIg levels than T3 tumors (Fig. 62c).

THE RESPONSE TO FRESHLY EXCISED AND CULTURED CCH1 TUMORS IN THE SAME MOUSE

The response to freshly excised and cultured CCH1 tumor cells were also compared by injecting these preparations into opposite limbs of the same mouse. In 10 mice, 1×10^6 excised CCH1 cells and 1×10^6 cultured CCH1 cells were injected into each of their hind right and left limbs respectively. On days 16 and 19, 5 mice were sacrificed and their tumors studied. On both days, excised CCH1 cells showed higher *in vivo* TAIg binding than cultured CCH1 cells, and they were found to produce larger tumors (Fig. 63). Moreover, TAIg binding on both types of tumors was found to increase with time.

THE RESPONSE TO DIFFERENT DOSES OF CCH_1 TUMOR CELLS IN THE SAME MOUSE

Four experiments were carried out to study the response to different doses of CCH1 tumor cells in the same mouse. Unlike the results obtained with similar studies in different mice where higher doses of tumor cells were found to produce higher *in vivo* TAIg binding (see 'application of the indirect RIAT'), conflicting results were obtained with experiments in the same mouse, where once the lower dose was found to produce the higher TAIg binding.

Ten mice in the first experiment were injected with 5×10^5 and 0.5×10^5 CCH1 tumor cells into their hind right and left limbs respectively. Tumors produced by the higher dose of tumor cells exhibited higher in vivo bound TAIg on day 17 and were larger (Figs. 64 and 65). By day 21, however, the TAIg gap between the 2 groups had appreciably decreased. Moreover, contrary to the general trend obtained in previous experiments, the TAIg binding was found to decrease with time in this experiment.

In a similar study in another experiment, tumors induced by different doses of tumor cells (5 versus 0.5×10^5 cells) in the same mouse were compared on day 20. As on day 22 in the previous experiment, no significant difference in TAIg binding was observed between the 2 groups (Fig. 66a). Tumors produced by the larger dose, however, were once again larger.

Contrary to the first experiment, the results of the third experiment showed higher in vivo TAIg binding on tumors produced by the lower dose of tumor cells (1 versus 0.1×10^6 cells) (Fig. 66b). Once again, however, the higher dose produced larger tumors.

Although no significant difference was obtained between the 2 groups in the fourth experiment, tumors produced by the higher dose (1 versus 0.2×10^6 cells) did show a slightly higher in vivo bound TAIg (Fig. 66c).

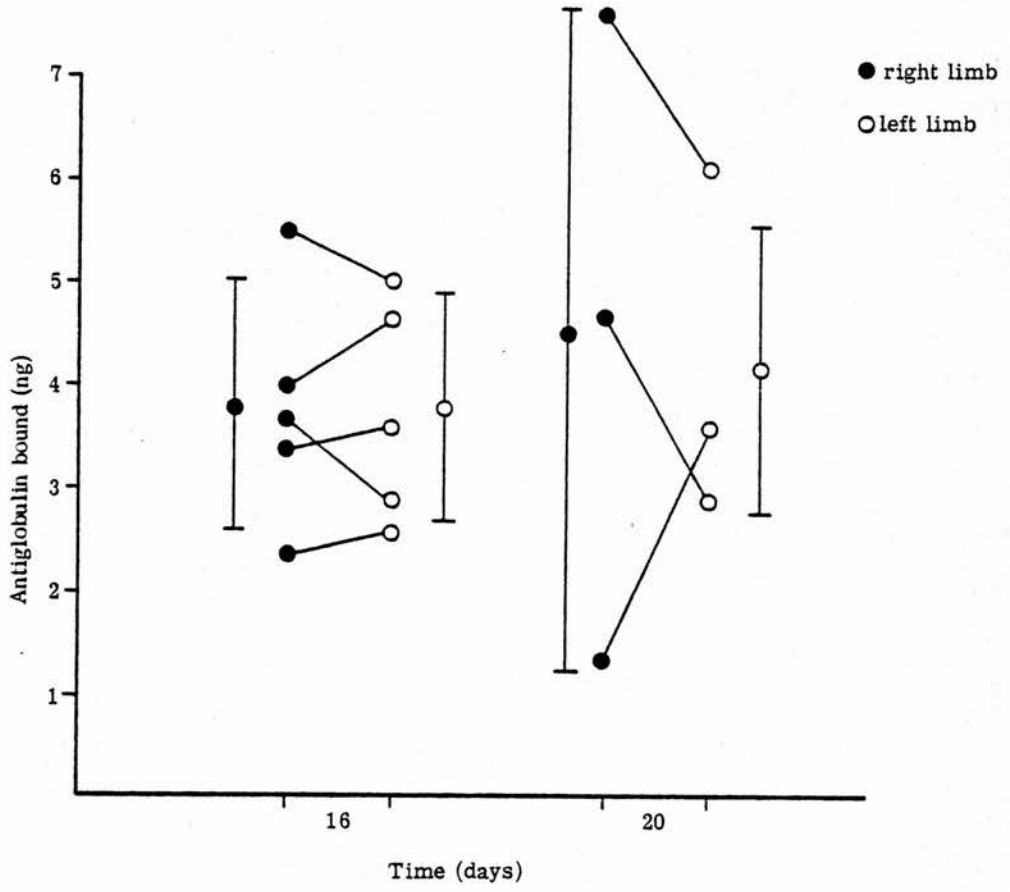


FIG 60 COMPARISON OF THE AMOUNT OF Ig BOUND TO CCH1 TUMORS OBTAINED FROM CONTRALATERAL SITES

CBA mice were injected s.c. in contralateral sites on Day 0 with 10^6 viable CCH1 tumor cells. On days 16 and 20, the amount of host Ig associated with 5×10^4 cells from individual tumors was assessed by the direct radio immune antiglobulin test. Note that no significant difference in TAIg was obtained between tumors grown on the right or left limbs of the mice.

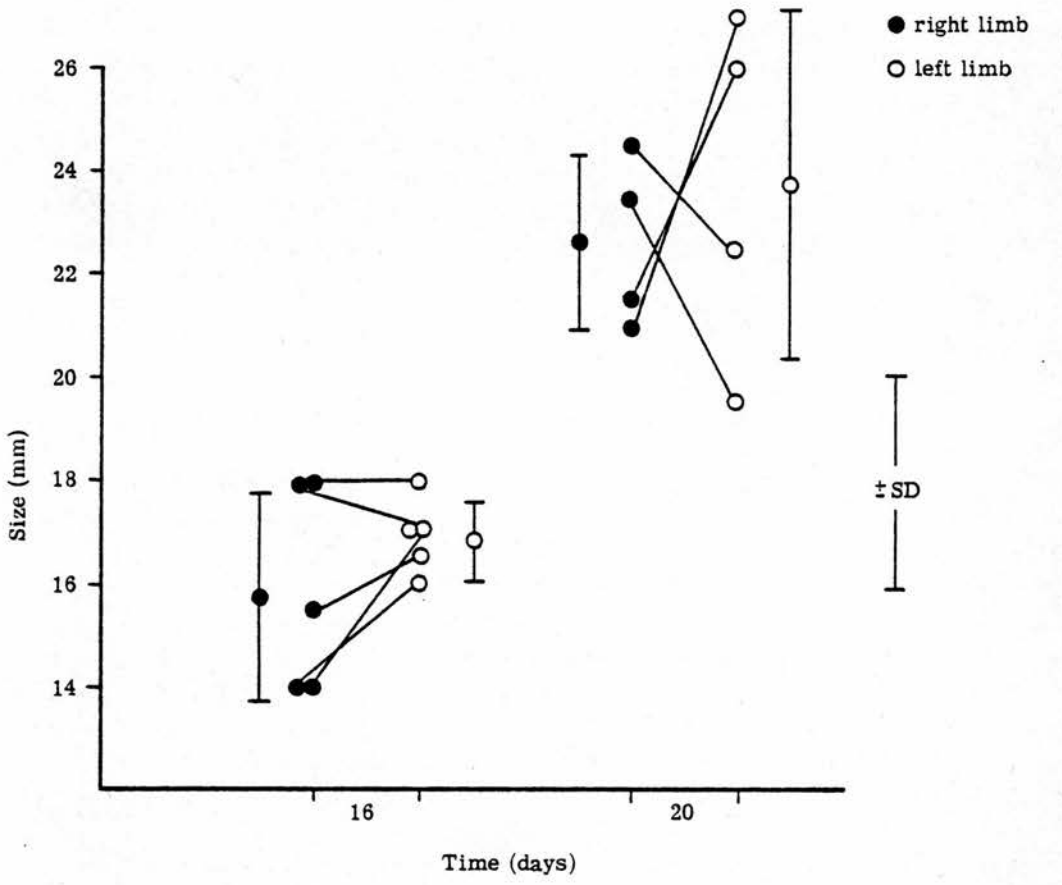


FIG 61 COMPARISON OF THE SIZE OF CCH1 TUMORS OBTAINED FROM CONTRALATERAL SITES

CBA mice were injected s.c. in contralateral sites on Day 0 with 10^6 viable CCH1 tumor cells. The figure depicts the sizes of the tumors on Days 16 and 20. Note that on either day, there is no significant difference in size between tumors grown on the right or left limbs.

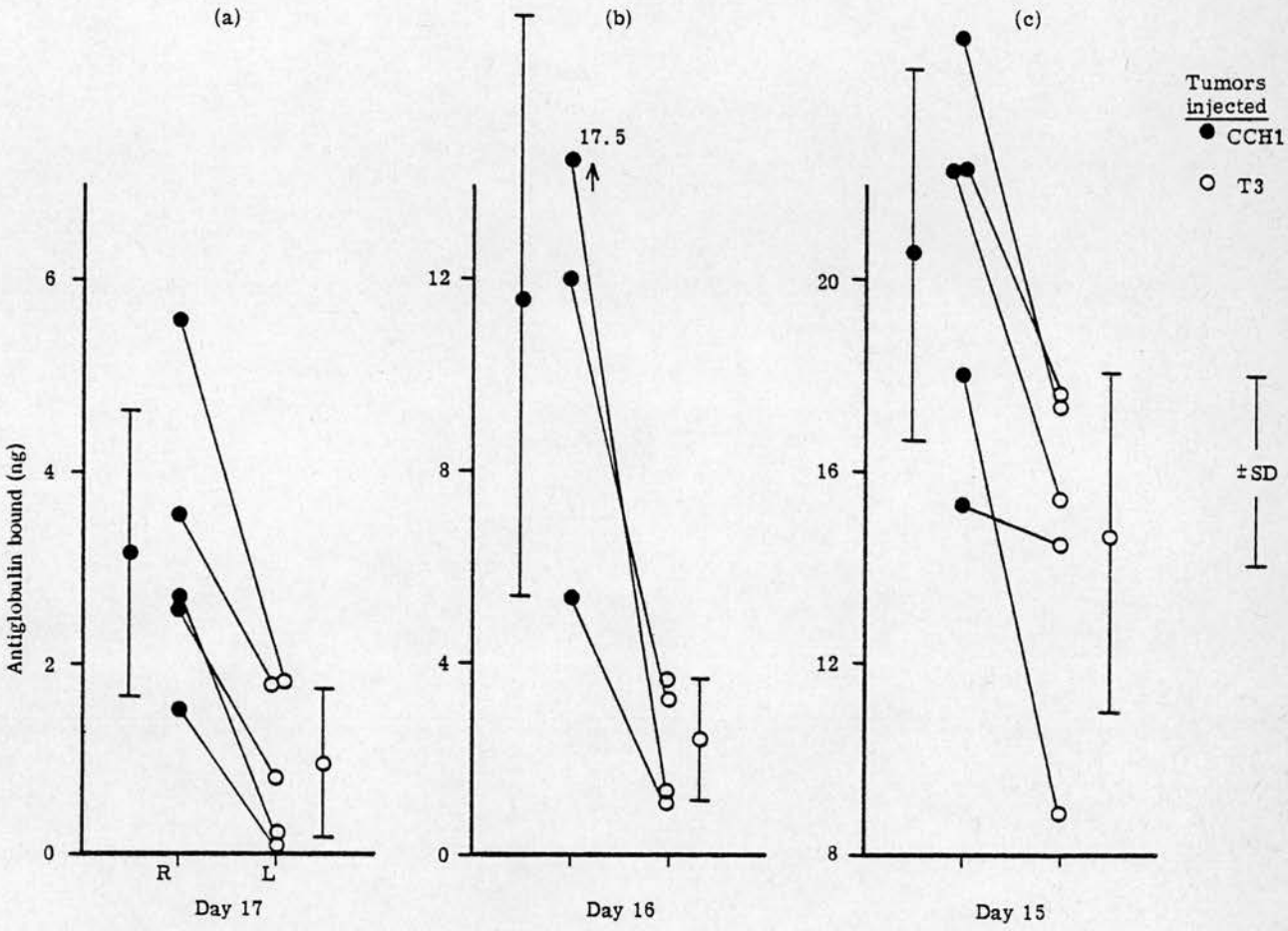


FIG 62 COMPARISON OF THE AMOUNT OF Ig BOUND TO CCH1 OR T3 TUMORS OBTAINED FROM CONTRALATERAL SITES.

CBA mice were injected s.c in contralateral sites on Day 0 with 10^6 viable CCH^1 or T3 tumor cells. About two weeks later, the tumors were excised and the amount of host Ig associated with 5×10^4 cells from individual tumors was assessed by the direct radio immune antiglobulin test. The figure represents the results of 3 separate experiments. Note that in all 3 experiments, CCH1 tumors exhibit appreciably higher TAIg than T3 tumors.

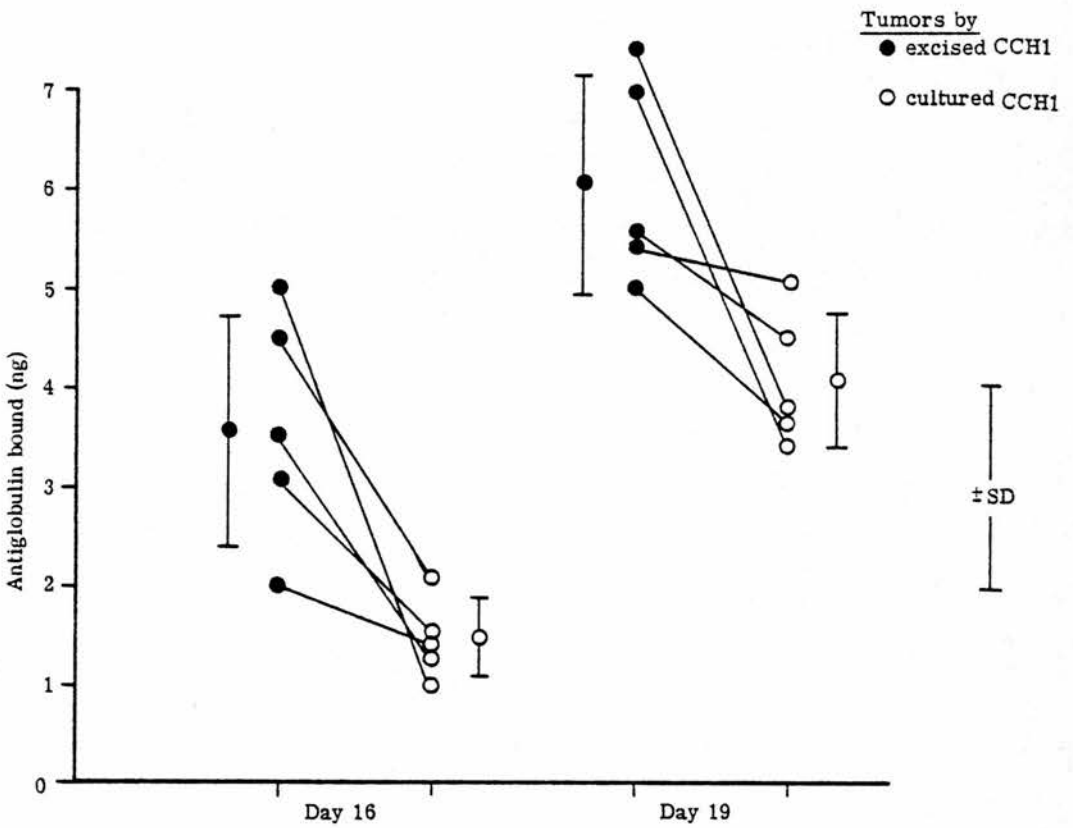


FIG 63 COMPARISON OF THE AMOUNT OF Ig BOUND TO TUMORS GROWN FROM FRESHLY EXCISED AND CULTURED CCH$_1$ TUMOR CELLS ON CONTRALATERAL SITES

CBA mice were injected s.c. in contralateral sites on Day 0 with 10^6 viable freshly excised or cultured CCH^1 tumor cells. On days 16 and 19, the amount of host Ig associated with 5 x 10^4 cells from individual tumors was assessed by the direct radio immune antiglobulin test. It can be observed that tumors grown from freshly excised tumor cells exhibited appreciably higher TAIg than tumors grown from cultured tumor cells. Note that TAIg binding increased with time in both types of tumors.

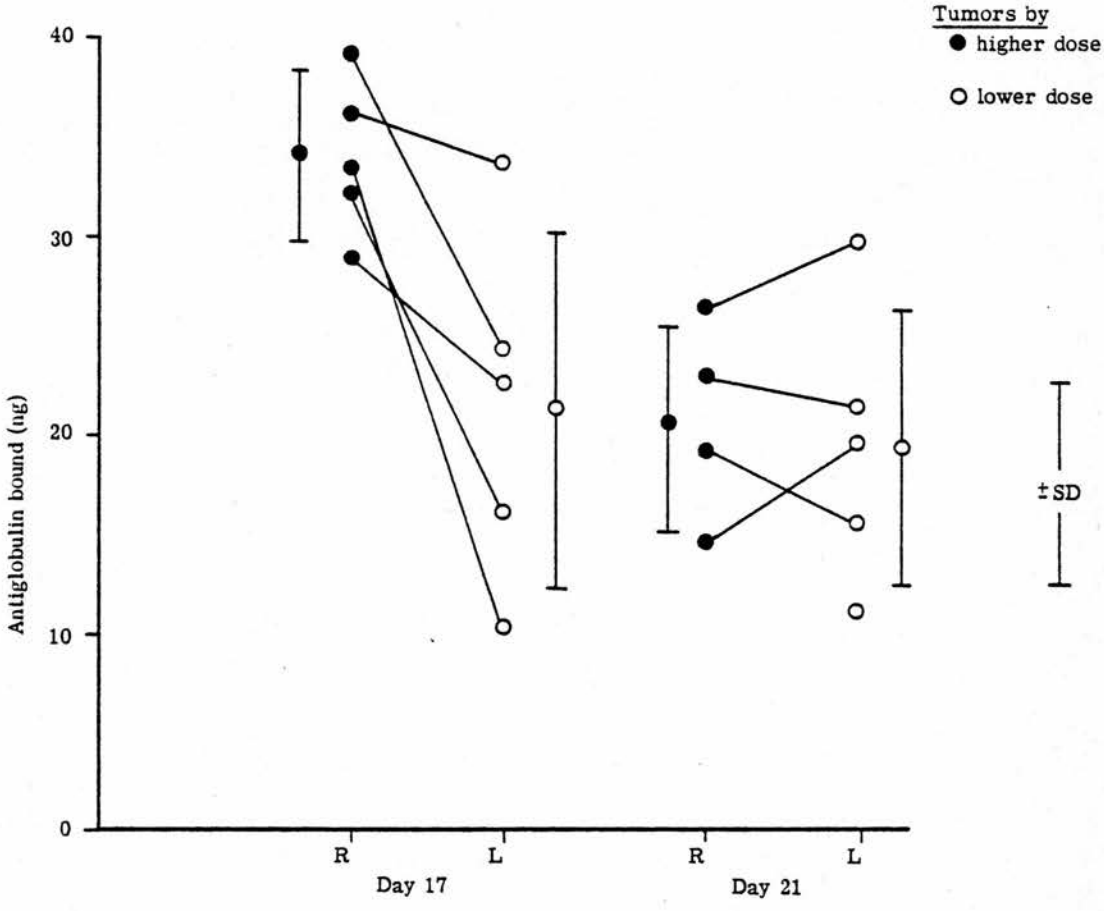


FIG 64 THE EFFECT OF TUMOR CELL DOSE ON TAIg BINDING

CBA mice were injected s.c. in contralateral sites on Day 0 with 5 or 0.5 x 10^5 viable CCH1 tumor cells. On days 17 and 21, the amount of host Ig associated with 5 x 10^4 cells from the individual tumors was assessed by the direct radio immune antiglobulin test. Note that on day 17, tumors produced by the higher dose of tumor cells exhibited higher TAIg amounts than tumors produced by the lower dose of tumor cells. However, this difference was no longer apparent on day 21 of tumor growth.

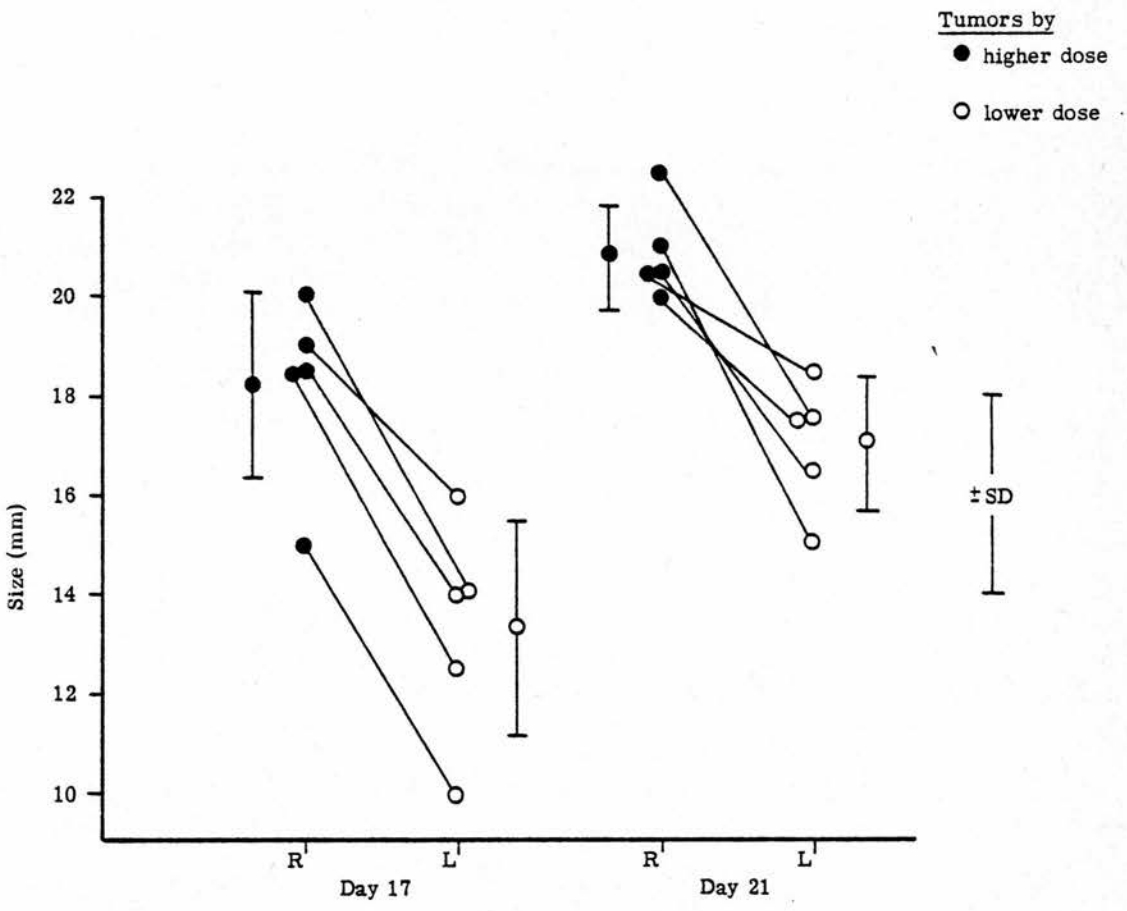


FIG 65 THE EFFECT OF TUMOR CELL DOSE ON TUMOR SIZE

CBA mice were injected s.c. in contralateral sites on Day 0 with 5 or 0.5 x 10^5 viable CCH1 tumor cells. The figure depicts the sizes of the tumors on Days 17 and 21. Note that on both days, tumors produced by higher dose of tumor cells were larger in size than the tumors produced by the lower dose of tumor cells.

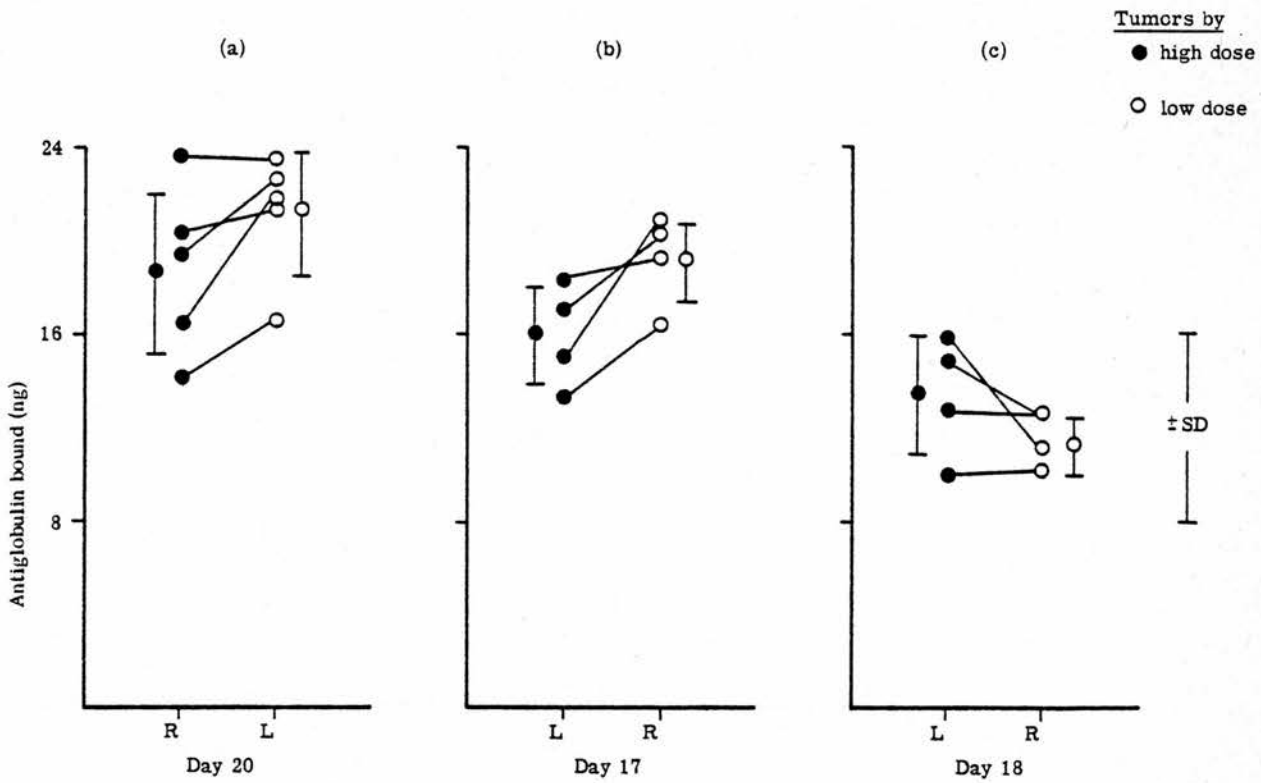


FIG 66 THE EFFECT OF TUMOR CELL DOSE ON TAIg BINDING

CBA mice were injected s.c. in contralateral sites on Day 0 with different doses of viable CCHI tumor cells. Two to three weeks later, the amount of host Ig associated with 5×10^4 cells from individual tumors was assessed by the direct radio immune antiglobulin test. The figure represents the results of 3 separate experiments.

a) Comparison between tumors produced by 5 or 0.5×10^5 CCH1 tumor cells. Note that there is no significant difference between the two groups. This result is similar to the observation made on Day 20 of tumor growth in Figure 64.

b) and c) Comparisons between tumors produced by 1 or 0.1 x 10^6 CCH_1 tumor cells. Note that there is no significant difference between the tumors produced by different doses.

ADDITIONAL C.PARVUM STUDIES

a) THE RESPONSE TO CCH1 WITH AND WITHOUT SC C. PARVUM TREATMENT

C. parvum studies were expanded to include tumors grown in the same mouse. The right limbs of the mice were subcutaneously injected with 0.2 ml (1.4 mg) C. parvum three days after the tumor injection, while the left limbs were not treated. As observed in Figure 67a, no significant difference in TAIg binding was observed between the 2 groups on days 17 and 21. Moreover, no inhibition of tumor growth was observed in C. parvum treated mice.

b) THE RESPONSE TO CCH1 WITH AND WITHOUT INTRATUMOR C. PARVUM IN THE SAME MOUSE

In order to study the effect of intratumor C. parvum treatment on in vivo TAIg binding, tumors growing in the left limb of the mice were injected with 0.2 ml (1.4 mg) C. parvum on day 8 following tumor injection, while tumors growing on the right limb were not treated. Once again, no significant difference was found between the 2 groups on days 18 and 22 (Fig. 67b). TAIg in both groups, however, increased with the age of the tumors.

SUMMARY

Results obtained with the direct RIAT were similar to the results exhibited by the indirect RIAT. CCH1 tumors showed higher *in vivo* bound TAIg than T3 tumors; excised tumor cells were found to bind higher *in vivo* TAIg than cultured tumor cells; and mice receiving ip *C. parvum* treatment exhibited higher *in vivo* bound TAIg on their tumors than non-treated mice.

New comparative experiments conducted in the same mouse reaffirmed the results obtained in different mice. CCH1 tumors were found to bind more *in vivo* TAIg than T3 tumors. Excised CCH1 tumor cells and larger CCH1 tumors in general, were found to bind higher *in vivo* TAIg than cultured CCH1 tumor cells and smaller CCH1 tumors respectively. However, SC or intratumor *C. parvum* treatment on days 3 and 8 respectively following tumor cell injection, was found not to influence tumor growth or the *in vivo* binding of TAIg.

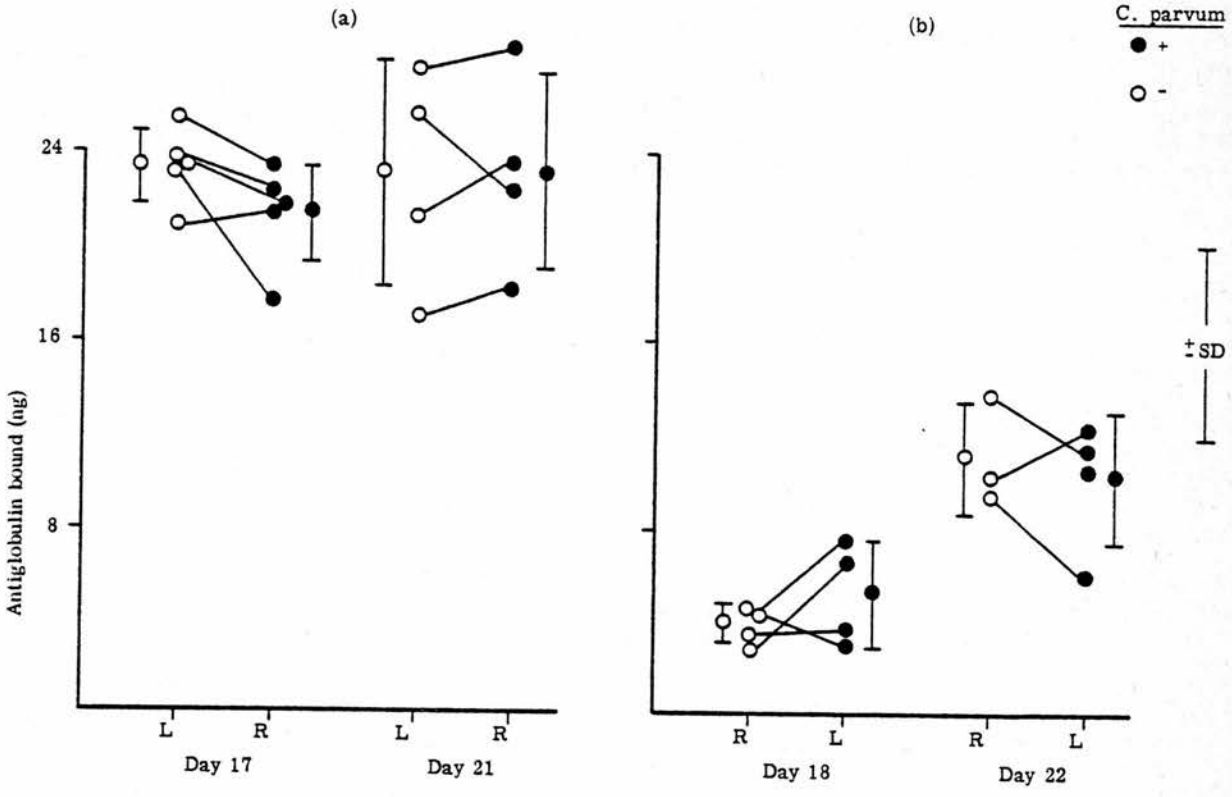


FIG 67 THE EFFECT OF LOCAL C.PARVUM TREATMENT ON TAIg BINDING

CBA mice were injected s.c. in contralateral sites on Day 0 with 10^6 viable CCH1 tumor cells. Tumors growing on one site were then treated with C.parvum, while tumors growing on the other sites were not treated. On various days thereafter, the amount of host Ig associated with 5 x 10^4 cells from individual tumors was assessed by the direct radio immune antiglobulin test. The figure represents the results of 2 experiments.

a) the right limbs of the mice were injected s.c. with 1.4 mg of C.parvum on day 3. It can be observed that no significant difference in TAIg binding was obtained between C.parvum treated and nontreated mice on days 17 and 21.

b) tumors growing on the left limbs of the mice were injected with 1.4 mg of C.parvum on day 8. No significant difference in TAIg binding between C.parvum treated or nontreated tumors was obtained on days 18 and 22. Note that in both groups TAIg binding increased with time.

No inhibition of tumor growth by C.parvum was observed in either experiment.

IMMUNOSUPPRESSIVE STUDIES

Immunosuppressive experiments were carried out in order to find out some of the mechanisms that might be involved in the in vivo binding of TAIgs. Nude mice and B mice were studied in order to determine the significance of T cells in the in vivo binding of TAIgs. The importance of macrophages in TAIg binding was investigated through the treatment of mice by gold salts. In addition, the effects of sublethal whole body irradiation on TAIgs was studied.

B mice consisted of thymectomized and X-irradiated mice. They were thymectomized at the age of 4-6 weeks, followed ten days later by whole body irradiation (850 rads) with thorax shielding. The mice were then allowed to recover over a period of several weeks. Controls included non-treated and sham thymectomized mice. T cell deprivation was assessed by visual examination for the absence of the thymus and by antibody response to SRBC. The right limbs of the mice were injected SC with 1×10^6 tumor cells around the age of twelve weeks.

Two experiments were carried out on B mice. In the first experiment, tumor growth and TAIg binding was examined on days 17 and 26 post tumor cell injection. Although tumors grown in normal mice were larger on day 17, no significant difference was observed in TAIg binding between normal and B mice on days 17 and 26 of tumor growth (Table 4). However, the significance of this experiment was limited by 2 criteria. Firstly, sham thymectomized mice were not tested; and secondly, the normal mice had been preinjected with CCH[1] cultured tumor cells iv 11 days prior to the SC injection of the tumor cells.

Sham thymectomized mice were included in the second experiment. On day 15 after the tumor cell injection, the tumors were excised and their TAIg content assessed. As can be observed from Table 4, no significant difference in TAIg levels was obtained between T-cell deficient and normal mice. However, sham thymectomized and thymectomized mice showed higher average levels of TAIgs in their tumors than nontreated mice. This could suggest that inflammatory reactions to the surgery may have led to the enhancement of antibody synthesis, some of which may then have non-specifically bound to the tumors.

Nude mice and their littermates were obtained from Moredun Institute, Edinburgh, and maintained in germ free conditions. Control mice included 12 week old CBA mice. The right limbs of the mice were injected with 1×10^6 tumor cells. The tumors were measured and excised on days 17 and 22, and the amount of TAIg present was assessed. Table 5 indicates that no significant difference in tumor growth or *in vivo* TAIg binding was obtained between the nude and CBA mice. The tumors did not grow in the littermates, which were not of CBA origin.

In order to study the effect of gold salt administration on TAIg binding, mice were injected 8 times intra-peritoneally with 1 mg sodium aurothiomalate. Gold-salt treatment has been suspected to effect macrophage function (James *et. al.* 1976). The treatment began on day 2 after the SC injection of 1×10^6 tumor cells per mouse, and continued at 2-3 day intervals (3 times a week). Tumors were measured and excised on day 24, and their TAIg content determined. Gold salt treatment was found to have no effect on tumor growth or *in vivo* binding of TAIg (Table 6).

Likewise, subjecting the mice to 400 rads whole body irradiation 24 hours before the injection of tumor cells had no major effect on TAIg binding (Table 7). However, the difference observed on day 21 approached

significance, especially when the increase of tumor size in irradiated mice was considered.

SUBCLASS STUDIES

Preliminary class and subclass studies were carried out in order to determine the quality of TAIg bound in tumors obtained from CP treated and nontreated mice. The indirect tube RAIT was applied in these studies. Briefly, the tumor cells were incubated with rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, or IgA for 1 hr on ice. After a few washes, the cells were incubated with ^{125}I-Swine anti-rabbit Ig for 1 hr on ice and then washed and counted.

In the two experiments that were carried out, CCH1 tumor cells were found to possess small quantities of all the classes and subclasses of the Igs tested (Table 8). Moreover, tumor cells from CP treated mice were found to have higher amounts of the Igs than tumor cells from non-treated mice, especially IgA, IgG2b, and IgG3.

TABLE 4 TUMOR-ASSOCIATED Ig LEVELS IN T-CELL DEPRIVED MICE

Exp.	Treatment	Day+ Examined	Ig extracted from 10^7 cells (ng)						Antiglobulin bound (ng)	SRBC response	
			M	A	G_1	G_{2a}	G_{2b}	G3		Total	Mercaptoethanol resistant
1	None X	17	5.7 ± 0.7	21.6 ± 4.6	2.5 ± 0.3	9.1 ± 0.9	<1	<3	3.0 ± 2.5	8.9 ± 1.2	6.2 ± 1.9
		26	6.3 ± 0.6	16.3 ± 6.9	2.1 ± 0.2	10.7 ± 1.1	<1	<3	1.9 ± 1.4		
	Thymecto-mized	17	5.7 ± 0.6	15.4 ± 3.2	2.3 ± 0.3	10.9 ± 0.3	<1	<3	1.7 ± 1.9	$4.6\pm1.9^*$	$0.9 \pm 1.2^*$
		26	6.4 ± 0.9	10.8 ± 2.0	2.0 ± 0.04	10.2 ± 0.9	<1	<3	1.5 ± 0.8		
2	None	15	NT	102.3 ± 38.9	3.7 ± 0.3	NT	NT	<3	8.5 ± 1.3	8.9 ± 0.7	7.4 ± 0.8
	Sham Thymecto-mized	15	NT	167.5 ± 99.5	3.7 ± 0.5	NT	NT	<3	10.0 ± 1.9	8.0 ± 0.5	6.3 ± 0.5
	Thymecto-mized	15	NT	127.7 ± 77.2	3.4 ± 0.4	NT	NT	<3	10.5 ± 1.6	$5.6\pm0.9^*$	$3.6 \pm 1.5^*$

+ Refers to the day tumor was removed. The anti-SRBC titres were determined 10 days after challenge

X Each group contains at least 4 mice

Note that thymectomy significantly inhibits the response to SRBC, but has a negligible effect on tumor-associated Ig levels.

TABLE 5 TUMOR-ASSOCIATED Ig LEVELS AND TUMOR SIZE IN NUDE MICE

Mice[X]	Day 17[+]		Day 22	
	Antiglobulin bound (ng)	Tumor size (mm)	Antiglobulin bound (ng)	Tumor size (mm)
normal	4.7 ± 1.7	13.8 ± 0.9	6.5 ± 3.1	13.5 ± 3.5
nude	5.7 ± 2.8	13.6 ± 2.5	6.3 ± 2.5	13.8 ± 1.7

+ Refers to the day tumor was removed

X Each group contains 3 - 4 mice.

TABLE 6 TUMOR-ASSOCIATED Ig LEVELS AND TUMOR SIZE IN GOLD-SALT TREATED MICE

Treatment[*X]	Day 24[+]	
	Antiglobulin bound (ng)	Tumor size (mm)
None	12.5 ± 5.2	23.8 ± 2.1
Gold-salt	9.5 ± 2.1	23 ± 1.8

\+ Refers to the day tumor was removed

X Treated mice were injected with sodium aurothiomalate (1mg) ip 8x, beginning on day 2 after s.c. injection of CCH_1 tumor cells. The mice underwent an ip injection every 3 days.

* Each group contains 4 mice.

TABLE 7 TUMOR-ASSOCIATED Ig LEVELS AND TUMOR SIZE IN IRRADIATED MICE

+X Treatment	Day 17*		Day 21	
	Antiglobulin bound (ng)	Tumor size (mm)	Antiglobulin bound (ng)	Tumor size (mm)
None	7.1 ± 2.7	16.3 ± 2.0	10.7 ± 3.7	16.3 ± 1.2
Irradiation	6.9 ± 2.9	14.9 ± 2.0	6.5 ± 3.2	16.9 ± 0.3

* Refers to the day tumor was removed

\+ Treated mice were irradiated (whole body, 400 rad) 24 hrs before the s.c. injection of CCH_1 tumor cells.

X Each group contains 3 - 4 mice

Note that although irradiation does not effect Ig levels on day 17, it appears to inhibit the Ig level on day 21.

TABLE 8 THE EFFECT OF C.PARVUM ON TAI $_{gs}$ AS ASSESSED ON THE TUMOR CELL-SURFACE

Exp.	C.parvum+ treatment	Antiglobulin bound (ng) on Day 21					
		M	A	G_1	G_2a	G_2b	G_3
1	No	0	0.6 ± 0.4	1.1 ± 1	0.9 ± 0.3	0	1.2 ± 0.4
	Yes	0.5 ± 0.6	2.8 ± 0.6*	1.8 ± 0.4	3.5 ± 2.0	2.1 ± 0.8*	2.9 ± 0.1*
2	No	0.9 ± 0.8	0.1 ± 0.1	0.8 ± 1.1	1.1 ± 0.4	0.8 ± 0.5	0.9 ± 0.1
	Yes	2.4 ± 0.7	3.4 ± 1.7*	1.9 ± 1.2	2.4 ± 1.3	3.2 ± 1.0*	3.5 ± 1.1*

+ 1.4 mg C.parvum injected ip 3 days after s.c. injection of 10^6 CCH1 tumor cells

Note that C.parvum treatment significnatly increases (*) the levels of Igs, especially IgA, Ig_2b, and IgG_3

D I S C U S S I O N

1. SUMMARY OF THE RESULTS

a. The development of the direct and indirect RIAT : The initial studies on the development of the isotopic antiglobulin test were carried out by the plate method. Preliminary results suggested the relevance of the indirect plate method for the semiquantitative assessment of *in vivo* bound TAIgs in freshly excised mechanically prepared tumor cells. It was observed that the sensitivity of the technique was diminished by non-specific binding of reagents to plastic and the binding of normal rabbit serum to tumor cells. The latter was suspected to be due to the presence of Fc-receptor bearing host cells in the tumor. The non-specific binding to plastic was inhibited by precoating the plates with 5% FCS-PBS overnight at 4 ^{o}C.

The sensitivity of the indirect RIAT was improved by the use of the tube method. This method permitted the use of larger volumes of washing media and facilitated the technique. Studies for optimal assay conditions indicated that the binding of the radio-iodinated antiglobulin reagent varied positively with reagent concentration, cell number, and incubation period. Further studies with the direct RIAT confirmed these results and the specificity of the method. The reagent was found to specifically bind to freshly excised tumor cells and spleen cells. Moreover, the binding of the reagent to tumor cells and host cells via their Fc-portion was found to be insignificant at the cell number used in our assay.

Enzyme studies with the direct and indirect RIAT demonstrated that the loss of TAIg from the surface of tumor cells increased with increasing enzyme concentration and incubation time. However, incubation of tumor tissues for half an hour at 37 ^{o}C with an enzyme concentration of 0.1 mg/ml

was found to yield sufficient number of viable cells without the removal of surface-bound TAIg.

b. The application of the direct and indirect RIAT : The RIAT was applied to the study of in vivo bound TAIgs and its variation with the age and size of tumors, the type of tumors, and the dose of tumor cells injected. The influence of non-specific immunostimulation and immunosuppression on the in vivo binding of TAIg were also examined. The indirect RIAT was used for the preliminary studies of TAIg. CCH1 tumors were found to contain higher amounts of TAIg than the less immunogenic T3 tumors. Similarly, tumors produced by freshly excised CCH1 cells were found to have more in vivo bound TAIg than tumors produced by cultured CCH1 tumor cells. Pronase prepared freshly excised tumor cells were demonstrated to produce tumors of higher TAIg amounts than tumors produced by mechanically prepared freshly excised tumor cells.

In general, the amount of in vivo bound TAIg was found to increase with the age and size of all types of tumors that were investigated. Moreover, larger doses of CCH1 tumor cells were shown to produce larger tumors which possessed higher amounts of TAIg than tumors produced by lower doses of CCH1 tumor cells. However, the positive relationship between in vivo bound TAIg and size of tumors was not absolute. Small tumors whose growth were inhibited by the administration of C.parvum were found to contain higher amounts of TAIg than the larger non-inhibited tumors.

Studies with the direct RIAT confirmed the above observations obtained by the indirect RIAT. Moreover, similar comparative studies were carried out on opposite limbs of the same mouse. As previously noted, CCH1 tumors were found to bind higher amounts of TAIg than T3 tumors. In addition, freshly excised CCH1 tumor cells and larger CCH1 tumors were

found in general to bind more TAIg than cultured tumor cells and smaller tumors respectively. SC or intratumor C. parvum treatment on days 3 and 8 respectively after tumor cell injection was not found to influence tumor growth or the in vivo binding of TAIg.

Preliminary immunoglobulin class and subclass studies with the indirect RIAT on CCH1 tumor cells indicated that in vivo bound TAIg was comprised of IgG1, IgG2a, IgG2b, IgG3, IgA and IgM. Moreover, tumors obtained from C.parvum treated mice were shown to contain significantly higher amounts of IgA, IgG3 and IgG2b. Parallel studies on CCH1 tumor extracts showed that tumors from C.parvum treated mice contained higher levels of IgA, IgG1 and IgM than tumors from nontreated mice.

T-cell deprivation was not found to influence the in vivo binding of TAIg. Tumors grown in thymectomized mice or nude mice were found to possess similar amounts of TAIg to tumors grown in normal mice. In addition, gold salt administration did not inhibit the binding of TAIg in CCH1 tumors. Likewise, whole body irradiation was not found to influence appreciably the in vivo binding of TAIg. However, tumors assessed on day 21 after tumor cell injection were observed to have lower TAIg than tumors obtained from non irradiated mice, although this was not significant. (For more information on these mechanism studies, refer to section 6 of discussion).

2. RELEVANCE OF THE RADIO IMMUNE ANTIGLOBULIN TEST TO THE STUDY OF TUMOR-ASSOCIATED IMMUNOGLOBULINS, AND ITS POTENTIAL

The radio immune antiglobulin test (RIAT) has been extensively investigated in order to ascertain its validity in studying cell-bound immunoglobulin. It has been primarily employed in measuring the *in vitro* binding by lymphoid cells and tumor cells of immunoglobulins from preparations believed to contain antibody. Our aim was to study Ig bound *in vivo* to the tumor by applying the RIAT on mechanically prepared tumor cells. The use of enzymes to obtain tumor cells was avoided as they were known to remove cell-surface bound proteins (Hynes, 1976).

The indirect RIAT which was used in our studies was based on an assay introduced by Harder and McKhann (1968). Similar to our studies, these authors and others (Sparks *et. al.* 1969) found that the uptake of the iodinated Ig was proportional to antibody concentration. The nonspecific binding which they observed was attributed to both radio-iodinated Ig and the test serum. Goldstein *et. al.* (1973) were able to detect by the RIAT non-cytolytic antibodies directed against cell surface antigens. Burdick *et. al.* (1973) obtained similar results with the RIAT demonstrating an almost linear relationship of the anti-globulin and test serum uptake with their corresponding concentrations. James *et. al.* (1977a) used the indirect RIAT to study anti-tumor anti-bodies in the sera of tumor bearing mice. Witz *et. al.* (1974) used a direct RIAT to study *in vivo* bound TAIgs in ascitic tumors.

Other investigations have employed iodinated staphylococcal protein A in the indirect RIAT. Protein A has been found to react with specifically bound mouse IgG, 2a, 2b, and 3; and human IgG 1, 2 and 4 (Kronvall *et. al.*, 1970). Brown *et. al.* (1977) used staphylococcal

protein A in order to study serum Igs produced against different tumors. As with the previous RIATs, the uptake of serum Ig was found to decrease with higher dilutions, but was still detectable at a dilution of 1/1600. When compared to the isotopic antiglobulin assay, the protein A method was found to be more sensitive. However, the former retained the advantage of detecting classes of Ig other than IgG.

While the RIAT has been widely used in the study of TAIg in ascitic tumors (Witz et. al. 1974; Dorval et. al. 1976; Braslawsky et. al. 1976a; Braslawsky et. al. 1976b and Moav et. al. 1978), its application to the study of TAIgs in solid tumors has been very limited. One of the main reasons behind this has been the difficulty of obtaining single cell suspensions of solid tumors without the use of enzymes. In our studies, the tumor cells were prepared mechanically either by gentle teasing or stirring of the tumor tissue, or by incubating the latter in collagenase (0.1 mg/ml) at 37°C . Cell surface proteins on tumor cells prepared by either method have been found to be qualitatively the same (Guy et. al. 1977). Although the cells prepared mechanically had low viability, the shape of the cells appeared intact, and the binding of the iodinated antiglobulin to the tumor cells was found to bear no relationship to their viability.

Enzyme studies by both the direct and indirect RIAT established that the loss of TAIg from the surface of freshly excised tumor cells was directly related to enzyme concentration and its time of incubation period. Although enzyme concentrations of 0.1 mg/ml achieved higher cell viabilities than the mechanical cell preparation, TAIg amounts on tumor cells prepared by either method were very similar.

Some of the factors which might have interfered with the sensitivity of both the direct and indirect RIAT include the following:

elution of TAIg; binding of reagents to plastic; loss of cells during the assay; and binding of the reagents to Fc-receptors on tumor and/or host cells. As previously mentioned (see 'Results' section), the effect of these factors wasminimized by various means. The elution of TAIg from the surface of tumor cells was avoided by performing the assay on ice or at 4°C; The nonspecific binding of the reagents to plastic was inhibited by precoating the tubes with 5-10 % FCS-PBS overnight at 4°C. The loss of cells was diminished by increasing the centrifugation time of each wash from three to five minutes, and by carrying out the assay in tubes instead of plates. Finally, while the binding of reagents to Fc receptors on the cells was found to occur at high cell concentrations, a similar binding of the reagents to Fc-receptors at the cell number used in our assay was found to be less significant.

As previously noted, our results also showed that reagent binding varied positively with reagent concentration, cell number, incubation time, and incubation temperature. Although the increase in temperature may have led to a limited elution of TAIgs from the surface of tumor cells, the results suggest that the latter must have been compensated for by increased binding of reagents to the TAIg on the cells due to higher kinetic energy at higher temperatures. However, an incubation temperature of 4°C was maintained in the direct RIAT in order to facilitate the comparison of its application with that of the indirect RIAT. Specificity studies by the direct RIAT demonstrated that reagent binding was restricted to freshly excised tumor cells, spleen cells, and cultured cells treated with anti-tumor antibodies. Thymus cells and cultured tumor cells did not bind the antiglobulin reagent.

It would appear, therefore, that the RIAT is relevant for the quantitative study of TAIgs. While the direct RIAT seems to be more

sensitive than the indirect RIAT, the specificity of both methods could be considerably improved by cell-separation techniques. The latter would involve the separation of host T-cells, B cells, macrophages, and dead cells from the freshly excised tumor cells. In addition, the specificity of the RIAT might also be further enhanced by multiple absorption of the reagents with cultured tumor cells and thymus cells. Finally, preliminary class and subclass studies have indicated that the indirect RIAT could also be suitable for qualitative studies of TAIgs.

With the improved assay conditions which are outlined above, the prospects for the use of the RIAT in tumor immunology research appear to be good. Radio iodinated immunoglobulins or Staphyloccocus aureus protein A have been shown to be sensitive enough to detect in vivo variation of TAIgs in tumors and the rebinding of eluted antibodies to cultured tumor cells. (Witz et. al., 1974; Dorval et. al., 1976; Braslawsky et. al., 1976b; Witz, 1977; Moav et. al., 1978 and Ehrlich and Witz, 1979).

Similar to our findings, variability in the in vivo TAIg binding between different tumors of the same group of mice has been observed (Dorval et. al., 1976). Moreover, this variability was found in the latter study and in some of our experiments to increase with the age of the tumors. Therefore, the observed variability among tumors of the same group seems to be due to different patterns of tumor progression in these tumors rather than being due to a defect in the RIAT. For example, different degrees of angiogenesis in different tumors within the same group could lead to variable TAIg binding. A study of the correlation between vascularization and TAIg binding should clarify this point.

The RIAT may also be exempt from another disadvantage that might be attributed to it; namely, the non specific binding of normal sera to

the cells. There is little doubt that the nonspecific binding of normal sera, and especially normal rabbit serum could be due to infiltrating Fc-receptor bearing host cells. (Szymaniec and James 1976; and Witz, 1977). The latter nonspecific binding can be diminished by the removal of Fc-receptor host cells. Moreover, such sera might bind to plastic thus decreasing the sensitivity of the RIAT. The use of siliconized glass tubes might improve the sensitivity of the RIAT.

Compared to other assay systems for TAIgs, such as immunofluoresence and haemadsorption, the RIAT appears to be well suited for the quantitative and qualitative study of TAIgs. It is objective, sensitive and economical.

3. RELATIONSHIP OF THE IMMUNOGENICITY AND DOSE OF TUMOR CELLS TO THE IN VIVO BINDING OF TAIg

The results showed a general tendency of the *in vivo* TAIg binding in tumors to vary positively with the immunogenicity of the tumor cells and their dose. CCH1 tumor cells were found to produce tumors of higher TAIg content than T3 tumor cells. Freshly excised CCH1 tumor cells and pronase prepared tumor cells were found to bind higher TAIg *in vivo* than cultured CCH1 tumor cells and mechanically prepared tumor cells respectively. In addition, larger doses of CCH1 tumor cells were shown to produce larger tumors containing higher amounts of TAIgs.

The higher *in vivo* binding of TAIg by CCH1 tumor cells compared to T3 tumor cells could be due to the higher immunogenicity of the CCH1 cells (James *et. al.* 1978). This hypothesis is particularly plausible if it is assumed that the majority of the TAIgs are anti-tumor antibodies.

On the other hand, if the TAIgs include nonspecific antibodies, then the lower in vivo binding of TAIg by T3 tumor cells may be due to its more rapid growth. In other words, it may be possible that those nonspecific antibodies are 'diluted' in the larger T3 tumors. The latter could also effect the binding of anti-tumor antibodies. Another reason for the lower amount of TAIg inT3 tumors could be due to the greater shedding of antigens by such tumors. This can be examined by assays for immune complexes in the sera of T3 tumor bearing mice. Such immune-complexes have been found to contribute to tumor growth by interrupting the anti-tumor immune response (Price and Robins, 1976).

A further reason for the lower TAIg binding by T3 tumor cells could be due to the production of proteolytic enzymes by these cells. Such enzymes may cleave the Fc portion of surface bound immunoglobulins (Keisari and Witz, 1976). In addition, since a constant number of cells were used in the RIAT, the decreased presence of TAIg on T3 tumor cells could be due to their slightly smaller size compared to CCH1 tumor cells. However, the latter hypothesis is unlikely especially that similar differences were noted in tumor cells of comparative sizes grown in vivo (i.e. CCH1 freshly excised vs cultued tumor cells). Finally, the observed differences between CCH1 and T3 tumor cells may be due to the influx of different types and numbers of host cells into the tumors (Pross and Kerbel, 1976), which may bind TAIg through their Fc-receptors (see also the addendum).

In an examination of the Ig subclass content in CCH1 and T3 tumor extracts, the latter were shown to have significantly lower levels of IgM and IgA (James et. al., 1979). This was further demonstrated in another experiment where T3 tumors were shown to contain significantly

lower levels of surface bound IgM. These observations could imply that the more immunogenic CCH1 tumor cells may induce complement binding antibodies which may inhibit their growth compared to T3 tumor cells. However, this has to be confirmed by further experiments on the sera of tumor bearing mice and their freshly excised tumor cells and tumor cell extracts. The differences of IgA binding between CCH1 and T3 tumors could be due to differences in the influx of polymorphonucleocytes which may possess receptors for the Fc-portion of IgA (Van Epps et. al., 1978). However, preliminary studies of host cell content in our tumors showed no significant differences between CCH1 and T3 tumors in the influx of PMN (see addendum).

Likewise, there could be many reasons to explain the higher in vivo binding of TAIg by CCH1 freshly excised tumor cells compared to cultured tumor cells. For example, freshly excised tumor cells may be more immunogenic than cultured tumor cells by the virtue of in vivo immunoselection. For the immunostimulation theory would predict that only the relatively immunogenic tumor cells would escape the immune response and propagate. Thus, freshly excised tumor cells may consist of a uniform population of tumor cells of high immunogenicity, while cultured tumor cells, which would have been multiplying in vitro for some time, could consist of a heterogenous population of cells of varying immunogenicity. Since, according to the immunostimulation theory again, higher immunogenicity may be correlated with enhanced tumor growth, then this could explain the faster growth of freshly excised CCH1 tumor cells and their greater binding of TAIg.

However, a more convincing hypothesis for the observed difference between cultured CCH1 tumor cells and their freshly excised counterpart

may be based upon the host cell content of the latter. Freshly excised tumor cells may contain plasma cells which could continue to produce anti-tumor antibodies when transplanted with the tumor cells. Moreover, freshly excised tumor cells may contain sensitized T-cells which could induce the influx of PMN by liberating lymphokines. A similar influx of antibodies and plasma cells could also be enhanced by macrophages which may facilitate tumor antigen processing. Moreover, another reason for the increased binding of TAIg by freshly excised tumor cells could be due to antigenic changes produced by the enzyme pronase. Enzymatic dissociation has been found to enhance the tumorigenicity of preneoplastic nodule lines (Medina et. al., 1978).

Quantitative differences of in vivo bound TAIg were also observed between freshly excised tumor cells prepared in different ways. Freshly excised pronase prepared CCH_1 tumor cells produced larger tumors containing higher in vivo bound TAIg than freshly excised mechanically prepared tumor cells.

Pronase preparation of tumor cells could have resulted in the following: the emergence of new antigenic sites; the production of more viable cells; the elimination of certain types of host cells; and/or the removal of surface bound TAIgs. These factors could have acted synergistically to enhance the influx of TAIgs into the tumors. However, the largest contribution of the enhanced immunogenicity of pronase prepared tumor cells could have come from the emergence of new antigenic sites (Bekesi and Holland, 1976; and Medina et. al., 1978), and the uncovering of old antigenic sites by the removal of surface-bound Igs. In contrast, the antigenic sites on mechanically prepared tumor cells would have remained covered by TAIgs, which could have diminished the immunogenicity of these cells. Such in vivo antigenic modulation has

been observed (Jacobson et. al., 1978). Moreover, although pronase and mechanically prepared tumor cells had an equal number of viable tumor cells, mechanically prepared cells were contaminated by a large number of dead cells, amongst which may have been dead host cells. Therefore, the lower number of viable host cells in the mechanically prepared cells may have hindered the influx of TAIg compared with pronase prepared cells.

The results in general indicated that the in vivo binding of TAIgs was dose dependent. This could imply a positive correlation between antigenicity and the inducement of TAIgs. Moreover, since larger doses of freshly excised tumor cells may contain a higher number of host cells, the observed difference between the different doses could be due to the host cell content of each dose of tumor cells. This can be tested by comparing different doses of cultured CCH_1 tumor cells.

4. IN VIVO KINETICS OF TAIgs

Studies with ascitic tumors have shown that the in vivo binding of TAIg increases with age of the tumors. Witz et. al. (1974) demonstrated that the in vivo binding of TAIg by various ascitic tumors increased on day 10 of tumor growth. Even though TAIg binding as measured by a direct RIAT was shown to remain constant until day 9, it was argued that TAIg binding was in fact increasing throughout this period. This was because, for example, the number of cells used in order to measure the TAIg on days 7 and 9 was 25×10^6 cells, while the total number of

tumor cells in the mouse had in fact increased from 290 to 550 x 10^6 cells from day 7 to 9. In other words, if TAIg binding to the whole tumor was indeed constant, then the amount of TAIg bound to 25 x 10^6 cells would have been expected to decrease from day 7 to day 9 due to the 'dilution' of TAIg by the size increase of the tumors. Therefore, there was a steady increase of TAIg binding with the age and size of the tumors. It was postulated by the authors that the latter paralleled the time-dependent increase in antibody synthesis following an immunogenic stimulus.

Likewise, Braslawsky et. al. (1976b) showed that the in vivo binding of TAIg by SEYF-a tumor cells increased during the first two weeks of tumor growth. These TAIgs were found to consist of complement dependent cytotoxic anti-tumor antibodies. Although the amount of TAIg in the tumor remained high during the third week of tumor growth, the anti-tumor antibodies were found to lose their cytotoxicity. The reason for this was shown to be due to cell-surface bound immune-complexes, which were present in close proximity to the anti-tumor antibodies.

Similar results were obtained on in vivo propagated SEWA, Ta3/St and YAC ascitic tumors (Dorval et. al., 1976). After an initial lag period, the in vivo binding of TAIg was found to increase considerably during the second week of tumor growth, reaching a plateau immediately prior to death of the mice. Witz and Moav (1978) demonstrated that IgG eluates from 11-15 day old SEYF-a tumor cells possessed the highest specific cytotoxic activity. This cytotoxic activity was found to decrease after the second week of tumor propagation, similar to a previous observation (Braslawsky et. al. 1976b).

A purpose of this study was to assess the variation of in vivo TAIg binding with the age and size of solid tumors. The studies were

carried out on tumors produced by freshly excised CCH1 and T3 tumor cells, and by cultured CCH1 and T3 tumor cells. While a general pattern of an increase in TAIg binding with age and size of CCH1 tumors was obtained, a similar pattern was not clearly observed with T3 tumors. In 7 out of 8 experiments on CCH^1 tumors, the in vivo TAIg binding was found to increase during the third week of tumor growth. Whereas in only 1 out of 4 experiments on T3 tumors the TAIg was shown to increase during the third week of tumor growth. However, when the overall data on TAIg was plotted against time, both CCH1 and T3 tumors exhibited a fluctuating pattern (see Figures 23 and 28). It was therefore postulated that the latter may have represented a rythmic shedding of immune complexes in vivo. This hypothesis was tested by the direct method, where the variation of TAIg binding with time in CCH1 and T3 tumors was studied every three days, from day 15 to day 30. Apart from an initial decrease of in vivo TAIg binding on day 18, both tumors showed a continuous increase of TAIg with time (see Figure 55). Similar to the above studies on ascitic tumors, the in vivo kinetics of TAIgs in solid tumors also appeared to parallel a time dependent increase in antibody synthesis following an immunogenic stimulus. The decrease of TAIg on day 18 could be due to the increase of tumor size without a similar increase in antibody production. However, this decline does not appear to last long, as antibody production and its binding to the enlarging tumor seems to increase from day 21. Alternatively, the decrease of TAIg on day 18 may represent shedding of immune-complexes from the surface of the tumor cells. Although in this case the shedding of immune-complexes would be expected to continue throughout tumor growth, the loss of TAIgs would appear to be rapidly made up by day 21 by the continuing synthesis of antibodies.

The results also indicated a general tendency of the TAIgs to increase with the size of tumors. Although this may have merely reflected the above mentioned increase of antibody synthesis with time, it may also have been due to increased angiogenesis in tumors with size (Folkman, 1978). Increased angiogenesis would be expected to increase the influx of anti-tumor antibodies and plasma cells into the tumors.

In conclusion, these findings suggest that antibody synthesis increases with time and that these antibodies bind to unoccupied antigens on old tumor cells and to the antigens on new cells produced by the rapidly propagating tumor. The specific and nonspecific binding of immunoglobulins may be enhanced by the increasing size and angiogenesis of the tumors. However, the in vivo kinetics of TAIgs remain complex and as discussed in section 6(c) a lot remains to be done in order to determine the nature of the TAIgs bound to tumors.

5. INFLUENCE OF C.PARVUM ON THE INVIVO BINDING OF TAIgs

C.parvum has been found to influence the humoral response in tumor bearing mice. James et. al. (1976) showed that the ip or sc administration of C.parvum led to increases in certain immunoglobulin levels and to the development of antibodies reacting with syngeneic and allogeneic tumor cells. The administration of gold salts was found to inhibit the observed effect of C.parvum, indicating therefore the importance of macrophages and PMN in the in vivo mechanisms of C.parvum

induced immune response. In another study, antibodies produced by C. parvum treatment were found to be extremely heterogeneous, some of which also reacted with syngeneic embryonic fibroblasts, and adult syngeneic kidney and spleen cells (James. et. al., 1977a).

Ip or iv administration of C. parvum was also found to induce increases in IgM in homozygous nude mice (nu nu), and IgM, IgG1, IgG21 and 2b in heterozygous nude mice (nu +) (James et. al., 1977b). Moreover, the ip administration of C.parvum was shown to increase anti-tumor antibodies too, though significantly so only in nu + mice. These results implied that the in vivo mechanisms of C.parvum induced immune response were partly T-independent.

Enhancement of antibody production has also been observed in tumor-bearing mice treated with other adjuvants like BCG. Complement-dependent cytotoxic anti-tumor antibodies were among the antibodies induced by BCG treatment (Boyle et. al. 1976). Macrophages, as well as T-cells, were also shown to be involved in the in vivo mechanisms of BCG induced immune response. Non-adherent cells in the presence of tuberculin antigen were found to cause the aggregation of cultured macrophage monolayers derived from BCG-sensitized mice (Preston et. al., 1977). In another study, MER was found to affect primarily the non-adherent cell population (Kedar et. al., 1978).

In our studies too, the administration of C.parvum was found to produce higher in vivo binding of TAIg both in CCH[1] and T3 tumors. In CCH1 tumors, the smaller C.parvum treated tumors were shown to possess higher amounts of in vivo bound TAIgs than the larger nontreated tumors. This was in contrast to the other findings which indicated that larger tumors usually tended to contain higher amounts of TAIgs. One of the reasons behind the latter could be due to the influx of different classes

and subclasses of antibodies, the possible *in vivo* mechanisms of which are expounded in section 6. Other reasons for the higher binding of TAIg in *C.parvum* treated tumors include the increased influx of macrophages, plasma cells, B cells and T cells into these tumors (see *addendum*). As mentioned above, *C.parvum* treatment has been suspected to enhance the activity of macrophages and T cells (James *et. al.* 1976), and their infiltration into tumors. It can therefore be speculated that the increased infiltration of macrophages and T cells would precipitate the increased influx of plasma cells and B cells, which in turn would raise the amount of TAIg bound by both tumor cells and host cells. Our results also indicated that the observed effect of *C.parvum* was dependent on the route and time of injection, for *C. parvum* injection sc or into the tumor on days 3 and 8 respectively was not found to inhibit tumor growth or enhance TAIg binding.

Different patterns in the variation of TAIg binding with time was obtained in *C.parvum* treated CCH1 and T3 tumors. While *in vivo* TAIg binding was found to increase with time in *C.parvum* treated CCH1 tumors, it was found to fluctuate in *C.parvum* treated T3 tumors. This result suggests that the inhibition of tumor growth by *C.parvum* treatment could be brought about by various means depending on the type of the tumor. For example, while certain TAIgs may be important in the growth inhibition of *C.parvum* treated CCH1, they may be less significant in *C.parvum* treated T3 tumors. The possible *in vivo* mechanisms of the *C.parvum* effects in CCH1 and T3 tumors is elaborated further in the following section.

6. MECHANISMS OF IN VIVO TAIg BINDING

a. The effect of T-cell deprivation

B mice and nude mice were used in order to study the effect of T-cell deprivation on the *in vivo* TAIg binding. Tumors were produced by injecting 1×10^6 CCH^1 tumors cells into the right limbs of mice.

In the first experiment, tumors grown in CBA normal and B mice were studied on days 17 and 26. Although tumor growth was found to be slightly inhibited in B mice on day 17, no significant difference was observed in the amounts of TAIg bound on days 17 and 26 (Table 4). It should be mentioned, however, that sham thymectomized mice were not used in this study. Furthermore, the normal mice had been injected with CCH^1 tumor cells iv 11 days prior to the SC injection of tumor cells.

Similar results were obtained in the second experiment where sham thymectomized mice were included. 15 day old tumors were excised and their TAIg content assessed. As can be seen from Table 4, *in vivo* TAIg binding was not found to be significantly effected by T-cell deprivation.

Additional studies in nude mice confirmed the results obtained in the above experiment. No significant difference in TAIg binding between normal and nude mice was observed on days 17 and 21 of tumor growth (Table 5). The tumors did not grow in the nude mice littermates.

It would therefore seem from these results that the *in vivo* binding of TAIgs may be T-independent.

b. The effect of gold salt treatment

Tumors grown in normal and gold-salt treated mice were compared for their *in vivo* TAIg binding. Treated mice were injected with 1 mg sodium auriothiomalate 8 times beginning on day 2 after the Sc injection

of 1 x 10^6 CCH1 tumor cells. As observed in Table 6, no difference was noted between gold-salt treated mice and non-treated mice. Since gold salts usually effect macrophages and PMN, this result would suggest therefore that the in vivo binding of TAIgs is independent of these cells.

c. The effect of sublethal irradiation

Mice were X irradiated (400 rads) 24 hrs before the SC injection of 1 x 10^6 CCH1 tumor cells. On day 17 of tumor growth, no significant difference in tumor size or TAIg binding was observed between normal and irradiated mice (Table 7). In contrast, on day 21, tumors grown in irradiated mice were found to have lower amounts of TAIg than tumors grown in normal mice. This was observed in spite of the fact that tumors in irradiated mice had increased in their size between days 17 and 21. Therefore, the difference observed on day 21 approached significance, especially when the increase of tumor size in irradiated mice was considered. This may have indicated a quantitatively decreased response of antibodies in irradiated mice. Moreover, since the tumors in the latter experiment were assessed on days 17 and 21, the results do not rule out the possibility of significant TAIg differences in younger tumors. Further studies are required in order to determine whether the in vivo TAIg binding is radiation sensitive. Our results were at variance with those obtained by Witz (1974) whose experiments suggested that TAIg binding by ascitic tumor cells was radiation sensitive. This discrepancy may have been due to the fact that the TAIgs in his experiments were assessed in younger tumors aged 5, 7 and 9 days. It would be of interest to find the relationship between sublethal irradiation, which effects T-suppressor cells, and the in vivo binding of TAIgs in solid tumors.

d. The effect of immunostimulation

This was studied by the ip administration of C.parvum (CP).

As mentioned previously, CP treatment was found to inhibit tumor growth and increase the quantitative binding of TAIg. Moreover, parallel studies on immunoglobulin class and subclass binding in tumor extracts showed CP treated tumors to possess significantly higher levels of IgA, as well as more IgM and IgG1 (Table 9). Similarly, two qualitative studies on surface-bound TAIgs in CP treated and nontreated tumors showed the former to possess higher levels of the immunoglobulin classes and subclasses tested (Table 8). Moreover, these differences were significant for IgA, IgG2b, and IgG3.

It would seem, therefore, that CP administration enhances the in vivo binding of most immunoglobulins to the tumor cells. Since a similar observation has been made on the humoral immune response in CP treated tumor-bearing mice, where a significant increase of IgG2b were noted, then our results could indicate that the elevated binding of TAIgs is a reflection of the elevated humoral response.

At present, the relationship of this increased humoral response and elevated TAIg binding to tumor growth inhibition in CP treated mice can only be speculative. Among the mechanisms that can be envisaged may be the cytolysis of tumor cells by complement-binding immunoglobulins such as IgG and IgM (this hypothesis is especially supported by recent results indicating that IgG1 can fix complement (Ey, 1979); the destruction of tumor cells by activated macrophages, which can be enhanced by opsonization; and enhanced T cell activity caused by antigen processing in activated macrophages. CP administration could also assist certain TAIgs to compete with immune-complexes for binding to host cells and tumor cells (Braslawsky et. al. 1976c).

TABLE 9 THE EFFECT OF C.PARVUM ON TAIgs AS ASSESSED IN TUMOR EXTRACTS

Exp.	C.parvum+ treatment	Day	Nos of tumors	Ig extracted from 10^7 cells (ng)					
				M	A	G_1	G_2a	G_2b	G_3
1	No	23	5	10.1 ± 1.2	26.3 ± 8.8	6.3 ± 0.9	16.2 ± 2.0	6.6 ± 0.8	NT
	Yes		5	$19.0\pm1.7^*$	$86.6\pm26.4^*$	6.3 ± 0.3	14.8 ± 0.8	7.6 ± 1.7	NT
	No	30	5	4.4 ± 0.4	18.0 ± 5.8	4.9 ± 6.9	15.8 ± 1.0	5.1 ± 0.9	NT
	Yes		4	$6.5\pm0.2^*$	$72.0\pm18.6^*$	$6.7\pm0.3^*$	18.4 ± 1.9	7.6 ± 1.7	NT
2	No	15	5	5.1 ± 0.4	31.7 ± 14.5	2.3 ± 0.6	12.4 ± 1.1	NT	NT
	Yes		1	6.8	64.0	3.6	14.2	6.0	NT
3	No	16	10	12.8 ± 0.9	29.1 ± 5.7	4.3 ± 0.8	12.6 ± 2.0	6.8 ± 1.0	< 3
	Yes		9	$15.9\pm0.9^*$	$66.6\pm19.4^*$	6.2 ± 0.9*	12.5 ± 1.1	7.1 ± 1.3	< 3
	No	28	3	11.8 ± 4.4	26.0 ± 15.4	5.5 ± 0.9	13.8 ± 2.0	5.8 ± 0.8	< 3
	Yes		8	14.6 ± 0.9	$42.1\pm12.4^*$	4.8 ± 1.0	13.1 ± 1.8	$12.6\pm5.5^*$	< 3

+ 1.4 mg C.parvum injected ip 3 days after s.c. injection of 10^6 CCH1 tumor cells

Note that C.parvum treatment significantly increases (*) the levels of Igs, especially IgA & $I_g M$

e. The nature of TAIgs

There are indications that TAIgs consist of antitumor antibodies and natural antibodies (Witz 1977). These TAIgs would be expected to bind to both tumor cells and host cells within the tumor. Antitumor antibodies would bind to tumor cells through their Fab portion, while natural antibodies, as well as the anti-tumor antibodies, could bind to host cells through their Fc-portion. Moreover, TAIgs may represent humoral antibodies or antibodies that are produced locally (Roberts *et. al.* 1973). In either case, the nature of TAIgs and the extent of their association with the different cells within the tumor remains to be determined.

Many strains of mice have been found to possess circulating anti-viral antibodies (Ihle and Hanna, 1977). Therefore, it was conceivable that TAIg detected in this study may consist of nonspecifically bound anti-viral antibodies. However, the sera of CBA mice used in our department have recently been shown to be devoid of such anti-viral antibodies (James *et. al.* 1978). This may have been due to the fact that the 10-12 week old mice routinely used in our studies were not yet expressing endogenous viral antigens. Tumor cell or *C.parvum* injections did not elicit antiviral responses. Moreover, there was no evidence of virus or virus-like particles in our cultured tumor cell lines when examined by electron-microscopy, nucleic acid incorporation studies, reverse transcriptase assays, or fluorescent studies with antisera to RD 114, MLV and anti-AKR P30 (James *et. al.* 1978). Therefore, it is unlikely that anti-viral antibodies constitute a part of the TAIgs detected in this study.

The fact that the *in vivo* binding of TAIgs depended on the immuno-

genicity of the tumor would tend to suggest that they consisted of antitumor antibodies. On the other hand, TAIgs may have been immunoglobulins bound merely to infiltrating host cells, for host cell infiltration has been also shown to depend on the immunogenicity of tumors: Eccles et. al. (1976) showed that the more immunogenic HSBPA tumors possessed significantly more monocytes than the less immunogenic MC3 tumors. Both of the latter tumors had been chemically induced. The issue may only be resolved when appropriate cell separation methods are carried out. Moreover, it has recently been shown that some strains of mice possess IgA and IgM autoanti-IgG antibodies (VanSnick and Masson, 1980). It can therefore be speculated that some of the TAIgs may be auto-anti Ig antibodies. This may explain the predominance of IgA in CCH1 tumors and needs to be investigated.

Two main effects can be envisaged regarding the role of TAIgs in vivo. These TAIgs can be tumor growth enhancers and/or tumor growth inhibitors. They could enhance tumor growth through immune-complexes which bind to immuno-competent host cells. Moreover, non-complement fixing antibodies which bind to tumor cells could prevent host cells from recognizing the tumor cell-surface antigens. On the other hand, the TAIgs could inhibit tumor growth through complement cytotoxicity and/or antibody-dependent cell cytotoxicity. Tumor cells have been shown to possess complement components ontheir surfaces, the activation of which may lead to chemotaxis of B cells, neutrophils, monocytes, and macrophages; as well as to increased vascular permeability. Moreover, TAIgs may facilitate macrophage binding to tumor cells.

Both of these contradictory effects of TAIgs could be operating simultaneously and the eventual outcome of tumor growth may depend on the nature of the TAIgs involved. Among the immunoglobulins whose levels were increased by CP administration were IgM and IgG1, IgG2b, and IgG3,

all of which bind complement. Although this is only a minor step towards correlating the role of TAIgs with its type, it gives credence to the attempts that are being made to determine the nature of TAIgs.

In conclusion, for a better understanding of the quantitative and qualitative assessments of TAIgs that were made in this study, the association of TAIgs with infiltrating host cells in CP treated and nontreated CCH[1] and T3 tumors should be determined, and the specificity and nature of immunoglobulins eluted from the tumor cells should be studied.

7. PROSPECTS OF TAIgs IN CANCER

As mentioned in the Introduction, TAIgs can be practically applied in tumor immunodiagnosis and immunotherapy. Although a lot remains to be done for the isolation and purification of anti-tumor antibodies, their significance in immunodiagnosis can be in little doubt. Anti-tumor antibodies, eluted from the surface of human tumor cells, can be concentrated, labelled with I^{131} or ^{125}I and reinjected into the patient to screen for occult metastatic sites. Moreover, anti-tumor antibodies thus eluted can be used for detecting soluble antigens inthe sera of cancer patients and normal people alike. They can also be used in Histology for determining tumor cells in a tissue specimen with the immunofluorescence microscope. However, Fc-receptors on infiltrating host cells in the latter tissue should be blocked by aggregated IgG prior to the addition of the anti-tumor antibodies.

Similar important applications of TAIgs can be envisaged for tumor immunotherapy. Eluted anti-tumor antibodies can be conjugated

to chemotherapeutic drugs in order to guide the latter to tumor cells. This would help avoid the side effects which these drugs incur upon the patient. Moreover, cytotoxic anti-tumor antibodies can be separated from non-cytotoxic blocking antibodies and reinjected regularly to post-operative patients in order to minimize metastatic spread.

It was found in this study that C.parvum (CP) administration increases the in vivo binding of TAIgs. Therefore, cancer patients can be injected with CP and serum samples, rich in anti-tumor antibodies, later obtained from them. Cytotoxic anti-tumor antibodies isolated from these sera and stored can then be used to inhibit metastatic spread inthe patient after surgery.

A D D E N D U M

Recent results obtained from studies on TAIgs that are continuing in our department are reported in this section. These include an assessment of the host cell content of tumors, a study of the nature of TAIgs, and determination of the characteristics of eluted TAIgs.

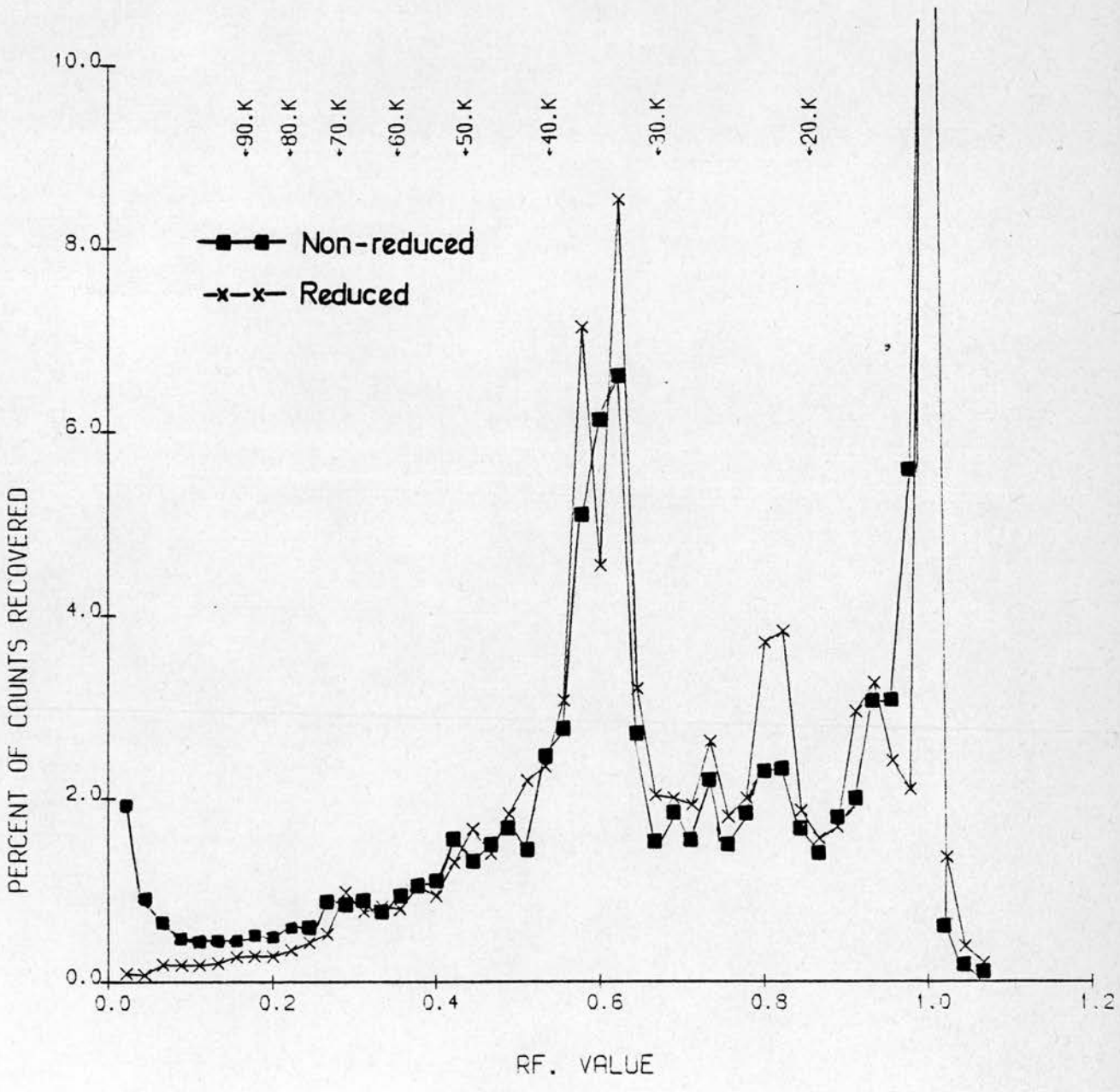
Preliminary studies on the host cell content of CCH1 tumors have shown that infiltrating host cells comprise 27-34% of the cells in tumors aged 14-23 days. Fc-receptor bearing cells were found to be the most prominent host cells, including lymphocytes, macrophages and PMN. A similar pattern of host cells in tumors obtained from CP treated mice was observed. However, infiltrating host cells in the latter were found to comprise 32-45% of the cells in tumors aged 18-35 days. Most of this increase in host cell content was found to be due to increased infiltration by Fc-receptor bearing cells and macrophages.

Infiltrating host cells in T3 tumors were found to comprise 20-37% of the cells in tumors aged 8-23 days. In contrast to CCH1 tumors, host cell content in T3 tumors was found to be evenly distributed between Fc-receptor bearing cells, lymphocytes and PMN. The macrophage content was consistently low. In general, CP treatment was not found to appreciably enhance host cell infiltration in T3 tumors. However, in one experiment on 18 day old tumors, CP treatment was found to appreciably increase host cell infiltration. Most of this increase in host cell content was observed to be due to increased infiltration by Fc-receptor bearing cells, lymphocytes and PMN. An increased influx of macrophages was also noted.

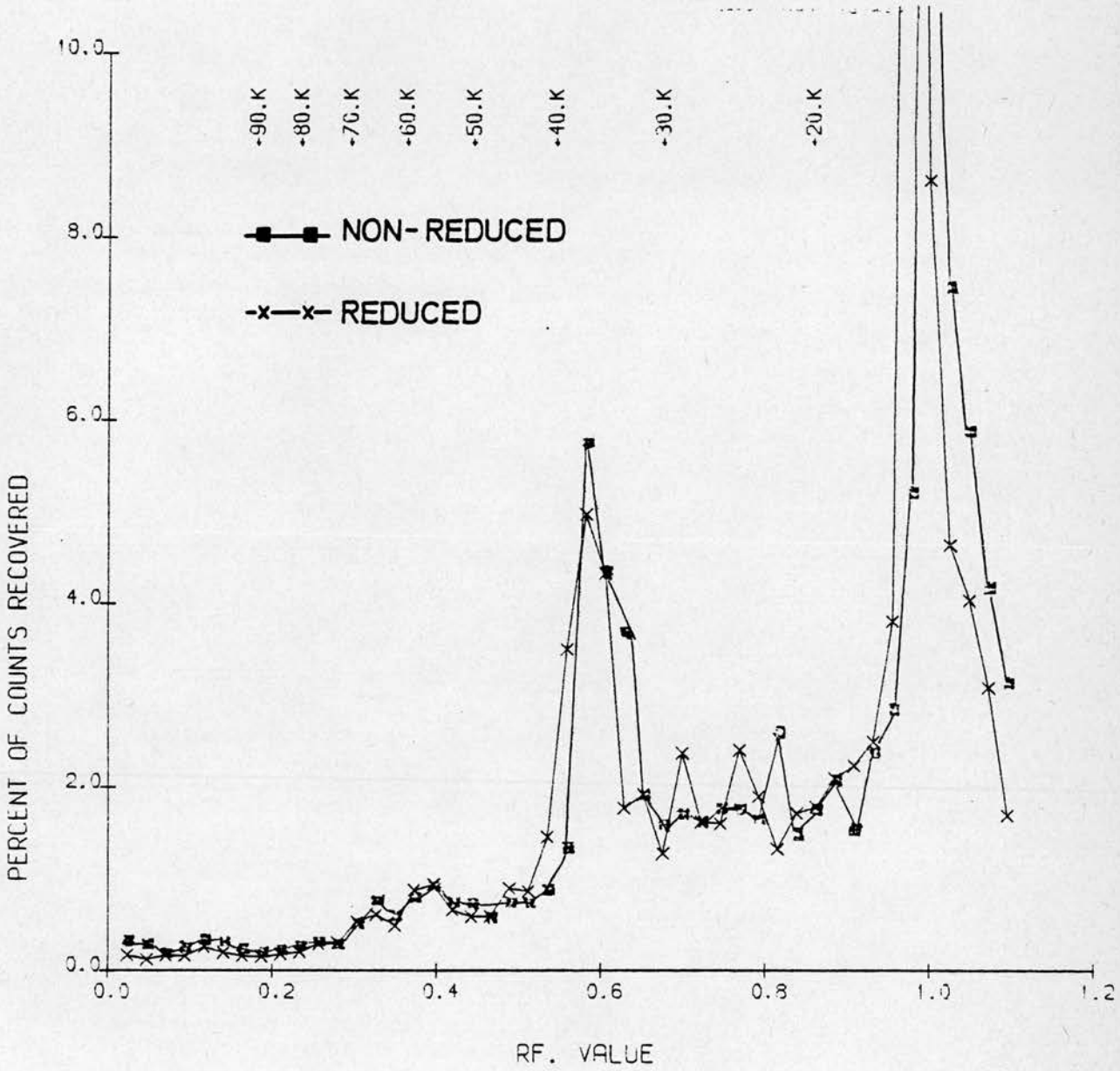
An attempt to determine the nature of TAIgs was made by pre-injecting the mice with SRBC. Since the CCH[1] tumors contained a high proportion of Fc-receptor bearing cells, it was expected that these cells may bind circulating anti-SRBC. Indeed, the results of 2 experiments showed that anti-SRBC antibodies comprised a part of TAIgs. For tumor cells obtained from mice which had been preinjected with SRBC were found to form significantly more rosettes with SRBC than tumor cells obtained from non-pretreated mice. Moreover, a similar observation was made with spleen cells from tumor-bearer or normal mice that had been pretreated with SRBC. These results suggested that some of the TAIgs may represent nonspecifically bound Igs to Fc-receptors on host and/or tumor cells. They may also represent Igs present on infiltrating sensitized lymphocytes and/or plasma cells.

Present studies on TAIgs also include the elution of these Igs and the determination of their characteristics. TAIgs are eluted by treatment of the tumor cells with acid citrate buffer or PBS. Preliminary studies with ^{125}I-labelled eluates have shown them to be heterogenous in nature. Although specific antibodies may be amongst the eluates, attempts to elute appreciable amounts of these specific antibodies have been unsuccessful so far. However, it is interesting to note that a major component in the eluate has been found to be a high molecular weight glycoportein. This component (33K) has been found in eluates from both freshly excised and cultured CCH[1] tumor cells. PAGE/SDS gel studies of extracts of tumors prelabelled by the lacto-peroxidase technique suggested that the 33K component was not well exposed on the surface of the intact tumor cells, for it was absent in such preparations. Additional studies indicated that the 33K component was in the form of a single polypeptide chain. Investigations are continuing on this component, especially in order to determine whether it represents an Fc-receptor.

PAGE - SDS GEL ANALYSIS OF ACID CITRATE ELUATE FROM CULTURED CCHI TUMOUR



PAGE - SDS GEL ANALYSIS OF ACID CITRATE ELUATE FROM FRESHLY EXCISED CCHI TUMOUR



REFERENCES

ALLISON, A.C., MONGA, J.N. and HAMMOND, V. 1974. Increased susceptibility to virus oncogenesis of congenitally thymus-deprived nude mice. Nature, 252, 746.

ASHBURN, P., COOPER, M.R., McCALL, E., and LAWRENCE, R.D. 1973. Nitroblue tetraziolium reduction: false positive and false negative results. Blood, 41, 921.

BAEHNER, R.L., NEIBURGER, R.G., JOHNSON, D.E. and MURRMANN, S.M. 1973. Transient bactericidal defect of peripheral blood phagocytes from children with acute lymphoblastic leukaemia receiving carniospinal irradiation. N. Eng. J. Med., 289, 1209.

BALDWIN, R.W. 1975. In vitro assays of cell-mediated immunity to human solid tumors: problems of quantitation, specificity, and interpretation. J. Natl. Cancer Inst., 55, 745.

BALDWIN, R.W., and PRICE, M.R. 1976. Immunobiology of rat neoplasia. Ann. NY Aca. Sci., 276, 3.

BALDWIN, R.W., HOPPER, D.G. and PIMM, M.V. 1976. Bacillus Calmette-Guerin contact immunotherapy of local and metastatic deposits of rat tumors. Ann. NY Aca. Sci., 277, 124.

BALDWIN, R.W. 1978. Immunological adjuvants in cancer immunotherapy. Develop. Biol. Standard, 28, 3.

BANSAL, S.C., HARGREAVES, R., and SJOGREN, H.O. 1972. Facilitation of polyoma tumor growth in rats by blocking sera and tumor eluates. Int. J. Cancer, 9, 97.

BAST, R.C., ZBAR, B., BORSOS, T. and RAPP, H.J. 1974. BCG and Cancer. N. Eng. J. Med. 290, 1413.

BAST, R.C., BAST, B.S. and RAPP, H.J. 1976. Critical review of previously reported animal studies of tumor immunotherapy with nonspecific immunostimulants. Ann. NY Aca. Sci., 277, 60.

BEAN, M.A., BLOOM, B.R., HERBERMAN, R.B., OLD, L.J., OETTGEN, H.F., KLEIN, G and TERRY, W.D. 1975. Cell-mediated cytotoxicity for bladder carcinoma: evaluation of a workshop. Cancer Res. 35, 2902.

BEKESI, J.G. and HOLLAND, J.F. 1977. Active immunotherapy in leukaemia with neuraminidase-modified leukaemic cells. Recent Results in Cancer Research, 62, 78.

BERD, D.A. and MITCHELL, M.S. 1976. Immunological enhancement of Leukaemia L120 by C.parvum in allogeneic mice. Cancer Res. 36, 4119.

BEVERLY, P.C.L. 1978. T effector cells. In. Immunological Aspects of Cancer. Castro, J.E., ed. pp 101-122. MTP Press Ltd., Lancaster, England.

BIRAN, H., MOAKE, J.L., REED, R.C., GUTTERMAN, J.V. HERSH, E.M. FREIREICH, E.J. and MAVLIGIT, G.M. 1976. Complement activation *in vivo* in cancer patients receiving *C.parvum* immunotherapy. *Br. J. Cancer*, 34, 493.

BLACK, M.N. and LEIS, H.P. 1971. Cellular responses to autologous breast cancer tissue. *Cancer*, 28, 263.

BOWEN, J.G., ROBINS, R.A. and BALDWIN, R.W. 1975. Serum factors modifying cell mediated immunity to rat hepatoma D23 correlated with tumor growth. *Int. J. Cancer*, 15, 640.

BOYLE, M.D.P., OHANIAN, S.H. and BORSOS, T. 1976. Detection of complement-dependent-antibody to tumor cells in sera of strain-2 guinea pigs cured of their tumors by BCG treatment. *J. Natl. Cancer Inst.*, 56, 623.

BRASLAWSKY, G.R., YAACKUBOWICZ, M., FRENDSDORFF, A. and WITZ, I.P. 1976a. Receptors for immune-complexes on cells within a nonlymphoid murine tumor. *J. Immunol.*, 116, 1571.

BRASLAWSKY, G., RAN, M. and WITZ, I.P. 1976b. Tumor-bound immunoglobulins: the relationship between the *in vivo* coating of tumor cells by potentially cytotoxic anti-tumor antibodies, and the expression of immune complex receptors. *Int. J. Cancer*, 18, 116.

BRASLAWSKY, G.R., SERBAN, D. and WITZ, I.P. 1976c. Receptors for immune-complexes on cells within a polyoma virus-induced murine sarcoma. *Eur. J. Immunol.*, 6, 579.

BROWN, J.P., KLITZMAN, J.M. and HELLSTROM, K.E. 1977. A microassay for antibody binding to tumor cell surface antigens using ^{125}I-labelled protein A from *staphylococcus aureus*. *J. Immunol. Methods*, 15, 57.

BRUCE, J., MITCHISON, N.A. and SHELLAM, G.R.1976. Studies on a gross-virus-induced lymphoma in the rat III. Optimization, specificity and applications of the *in vitro* immune response. *Int. J. Cancer* 17, 342.

BURDICK, J.I., COHEN, A.M. and WELLS, S.A. 1973. A simplified isotopic antiglobulin assay: detection of tumor cell antigens. *J. Natl. Cancer Inst.*, 50, 285.

BURNET, F.M. 1970. The concept of immunological surveillance. *Prog. Exp. Tumor Res.*, 13, 1.

CANTRELL, J.L., KILLION, J.J.and KOLLMORGEN, G.M. 1976. Correlations between humoral immunity and successful chemotherapy-immunotherapy. *Cancer Res.*, 36, 3051.

CARTER, S.K. 1976. Immunotherapy of cancer in Man: current status and prospectus. *Ann. N.Y. Acad Sci.*, 277, 722.

CASPI, R. and WITZ, I.P. 1976. The effect of short-term culture on the sensitivity of MDAY murine tumor cells to lysis mediated by normal rabbit serum. Eur. J. Immunol., 6, 174.

CHAR, D.H., HOLLINSTEAD, A., COGAN, D.G., BALLINTINE, E.J., HOGAN, M.J and HERBERMAN, R.B. 1974. Cutaneous delayed hypersensitivity reactions to soluble melanoma antigen in patients with ocular malignant melanoma. N. Engl. J. Med., 291, 274.

CHIRIGOS, M.A. 1976. The effect of immune modulators on the oncogenic process: adjuvant effect of staphylococcus aureus in treating murine leukaemia. Ann. NY Acad. Sci., 276, 513.

CLINE, M.J. 1973. A new white cell test which measures individual phagocyte function in a mixed leucocyte population I. a rentrophil defect in acute myelocytic leukaemia. J. Lab. Clin Med., 81, 311.

COGGIN, J.H., AMBROSE, K.R., DIERLAM, P.J. and ANDERSON, N.G. 1974. Proposed mechanisms by which autochthonous neoplasma escape immune rejection. Cancer Res., 34, 2092.

CONLEY, F.K. and REMINGTON, J.S. 1978. Effect of corynebacterium parvum on tumor growth in the central nervous system of mice. J. Natl. Cancer Inst., 61, 827.

COOPER, N.R., POLLEY, M.J. and OLDSTONE, M.B.A. 1974. Failure of terminal complement components to induce lysis of moloney virus transformed lymphocytes. J. Immunol., 112, 866.

DAUPHINE, M.J., TALAL, N., and WITZ, I.P. 1974. Generation of non-complement fixing, blocking factors by lysosomal extract treatment of cytotoxic anti-tumor antibodies. J. Immunol. 113, 948.

DOMZIG, W and LOHMANN-MATHES, M.L. 1979. Antibody-dependent cellular cytotoxicity against tumor cells. II. the promonocyte identified as effector cells. Eur. J. Immunol., 9, 267.

DORSETT, B.H., IOACHIM, H.L., STOLBACH, L., WALKER, J., and BARBER, H.R.K. 1975. Isolation of tumor-specific antibodies from effusions of ovarian carcinomas. Int. J. Cancer, 16, 779.

DORVAL, G., WITZ, I.P. KLEIN, E. and WIGZELL, H. 1976. Tumor-bound immmunoglobulins I. Further analysis of the characteristics of binding of immunoglobulins to in vivo grown tumors. Int. J. Cancer 17, 109.

ECCLES, S.A., BANDLOW, G. and ALEXANDER, P. 1976. Monocytes associated with the growth of transplanted syngeneic rat sarcomata differing in immunogenicity. Br. J. Cancer, 34, 20.

EHRLICH, R. and WITZ, I.P. 1979. The elution of antibodies from viable murine tumor cells. J. Immunol. Methods, 26, 345.

EVANS, C.H., OHANIAN, S.H. and COONEY, A.M. 1975. Tumor-specific and forssman antigens of guinea-pig hepatoma cells: comparison of tumor cells grown in vivo and in vitro. Int. J. Cancer, 15, 512.

EY, P.L., PROWSE, S.J. and JENKIN, C.R. 1979. Complement-fixing IgG1 constitutes a new Ig subclass of mouse IgG. Nature, 281, 492.

FARRAM, E., FESTERSTEIN. H., and DEGIORGI, L. 1978. The role of antibody in the inhibition of the growth of meth. A. tumor in syngeneic experiments in vivo and in vitro. Clin. Exp. Immunol. 33, 377.

FLORES, M., MARTI, J.H., GROSSER, N., MACFARLANE, J.K. and THOMSON, D.M.P. 1977. An overview: Anti-tumor immunity in breast cancer assayed by tube leukocyte adherence inhibition. Cancer, 39, 494.

FOLKMAN, J. 1978. Tumor angiogenesis and tumor immunity. In, Immunological Aspects of Cancer. Castro, J.E., ed. p. 267. MTP Press Ltd., Lancaster, England.

FREEMAN, C.B. 1978. Immunotherapy of leukaemia. In, Immunological Aspects of Cancer. Castro. J.E., ed. p. 385 MTP Press Ltd., Lancaster, England.

FRIDMAN, W.H., NELSON, R.A. and LIABEUF, A. 1974. Production of an immunoglobulin-binding-factor (IBF) by antigen-stimulated lymphnode lymphocytes. J. Immunol., 113, 1008.

FRIEDMAN, H. and CEGLOWSKI, W.S. 1975. Immunosuppression in the etiology of cancer. In, Immunological Aspects of Neoplasia. p. 253. The Williamson and Wilkins Co., Baltimore, Maryland.

FRIEDMAN,. H. SPECTER, S., KAMO, I. and KATELEY, J. 1976. Tumor-associated immunosuppressive factors. Ann NY Acad Sci., 276, 417.

FUJIMOTO, S., GREEN, M.I. and SEHON, A.H. 1976a. Regulation of the immune response to tumor antigens. I. immunosuppressor cells in tumor-bearing hosts. J. Immunol., 116, 791.

FUJIMOTO, S., GREEN, M.I. and SEHON, A.H. 1976b. Regulation of the immune response to tumor antigens. II. Nature of immunosuppressor cells in tumor-bearing hosts. J. Immunol., 116, 800.

GERSTH, B., ENG., L.F. and BIGBEE, J.W. 1977. Tumor-associated immuno-globulins in pulmonary carcinoma. Cancer Res., 37, 4449.

GHOSE, T., TAI, J., GUOLU, A., NORVELL, S.T., BODURTHA, A., AQUINO, J. and MACDONALD, A.S. 1976. Antibodies as carriers of radionuclides and cytotoxic drugs in the treatment and diagnosis of cancer. Ann NY Acad Sci., 277, 671.

GHOSE, T. and Blair, A.H. 1978. Antibody-linked cytotoxic agents in the treatment of cancer: current status and future prospects. J. Nat. Cancer Inst., 61, 657.

GOLDROSEN, M.H. and DENT, P.B. 1977. Characterization of immunoglobulins eluted from hamster SV40 tumors. Immun. Communications, 6, 133.

GOLDSTEIN, L.T., KLINMAN, N.R. and MANSON, L.A. 1973. A microtest radioimmunoassay for noncytotoxic tumor-specific antibody to cell surface antigens. J. Natl. Cancer Inst., 51 1713.

GUPTA, R.K. and MORTON, D.L. 1975. Suggestive evidence for in vivo binding of specific antitumor antibodies of human melanomas. Cancer Res., 35, 58.

GUPTA, R.K., SILVER, H.K.B., REISFELD, R.A. and MORTON, D.L. 1979. Isolation and immunochemical characterization of antibodies from the sera of cancer patients which are reactive against human melanoma cell membranes by affinity chromatography. Cancer Res., 39, 1683.

GUTTERMAN, J.V., MAVLIGIT, G.M., BLUMENSTEIN, M.A.B., MCBRIDE, C.M. and HERSH, E.M. 1976. Immunotherapy of human solid tumors with Bacillus Calmette-Guerin: prolongation of disease free interval and survival in malignant melanoma, breast, and colorectal cancer. Ann NY Acad. Sci., 277, 135.

GUTTERMAN, J.V., MAVLIGIT, G.M. SCHOWZ, M.A. and HERSH, E.M. 1978. Immunotherapy of human solid tumors:principles of development. In, Immunological Aspects of Cancer. Castro, J.E. ed. p. 415. MTP Press Ltd., Lancaster, England.

GUY, D., LATNER, L. and TURNER, A. 1977. Radioiodination studies of tumor cell-surface proteins after different disaggregation procedures. Br. J. Cancer, 36, 166.

HANSEN, J.A. and GOOD, R.A. 1974. Malignant disease of the lymphoid system in immunological perspective. Human Pathology, 5, 567.

HARDER, F.H. and MCKHANN, C.F. 1968. Demonstration of cellular antigens on sarcoma cells by an indirect I-labelled antibody technique. J. Natl. Cancer Inst., 40, 231.

HARRIS, T.N., HARRIS, S., HENRI, E.M. and FARBE , M.B. 1978. Enhancement of growth of allogeneic mouse tumor by the IgG_1 fraction of allo-antibody preparations. J. Natl. Cancer Inst., 60, 167.

HASKILL, J.S., YAMARRURA, Y., RADOV, L., and PARTHENAIS, E. 1976. Discussion paper: are peripheral and in situ tumor immunity related ? Ann. NY Acad Sci., 276, 373.

HASKILL, J.S. 1977.ADCC effector cells in a murine adeno-carcinoma I. Evidence for blood-borne bone-marrow derived monocytes. Int. J. Cancer, 20, 432.

HASKILL, J.S. RADOV, L.A., FETT, J.W. and PARTHENAIS, E. 1977. The antibody response to the T1699 murine adenocarcinoma: antibody class and subclass heterogeneity detected in serum and in situ. J. Immunol., 119, 1000.

HEIDRICK, M.L., RYAN, W.L. and CURTIS, G.L. 1978. Stimulation of malignant skin cells by antibody to normal skin cells of mice. J. Natl. Cancer Inst., 62 1273.

HEIMEN, R., and KLEIN, E. 1976. Circulating immune complexes in sera of patients with Burkitt's lymphoma and nasopharyngeal carcinoma. Int. J. Cancer, 18, 310.

HELLSTROM, I., HELLSTROM, K.E., SJOGREN, H.O. and WARNER, G.A. 1971. Demonstration of cell-mediated immunity to human neoplasms of various histological types. Int. J. Cancer, 7, 1.

HERBERMAN, R.B., NUNN, M.E., HOLDEN, H.T. and LARVIN, D.H. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors II. characterization of effector cells. Int. J. Cancer, 16, 230.

HOFFKEN, K., PRICE, M.R., MCLAUGHLIN, P.J., MOORE, V.E. and BALDWIN, R.W. 1978a. Circulating immune complexes in rats bearing chemically induced tumors. I. Sequential determination during the growth of tumors at various body sites. Int. J. Cancer, 21, 496.

HOFFKEN, K., PRICE, M.R., MOORE, V.E. and BALDWIN, R.W. 1978b. Circulating immune complexes in rats bearing chemically induced tumors II. characterization of sera from different stages of tumor growth. Int. J. Cancer, 22, 576.

HOFFKEN, K., MCLAUGHLIN, P.J., PRICE, M.R., PRESTON, V.E. and BALDWIN, R.W. 1978c. Rat C_{1q}: similarity to human C_{1q} in functional and compositional properties. Immunochemistry, 15, 409

HOLDEN, H.T., HASKILL, J.S., KIRCHNER, H., and HERBERMAN, R.B. 1976 Two functionally distinct anti-tumor effector cells isolated from primary murine sarcoma virus-induced tumors. J. Immunol., 117, 440.

HOLLAND, J.M, MITCHELL, J.J., GIPSON, L.C. and WHITAKER, M.S. 1978. Survival and cause of death in ageing germ free athymic nude and normal inbred C3Hf/$_{He}$ mice. J. Natl. Cancer Inst., 61, 1357.

HIRS AUT, Y., PINSKY, C.M., WANEBO, H.J. and OETTGAN, H.F. 1976. Design of phase-I trials of immunopotentiators for cancer therapy: levamisole and Corynebacterium parvum: Ann NY Acad Sci., 277, 252.

HYNES, R.O. 1976. Cell surface proteins and malignant transformation. Biochimica et Biophysica Acta, 458, 73.

IHLE, J.N. and HANNA M.G. 1977. Natural immunity to endogenous Oncornaviruses in mice. In, Contemporary Topics in Immunobiology. Hanna, M.G. and Rapp, F. Eds. p. 169. Plenum Publishing Corporation, New York.

IRIE, K., IRIE, R.F. and MORTON, D.L. 1974. Evidence for in vivo reaction of antibody and complement to surface antigens of human cancer cells. Science, 186, 454.

IRIE., K., IRIE, R.F. and MORTON, D.L. 1975. Detection of antibody and complement complexed in vivo on membranes of human cancer cells by mixed hemadsorption techniques. Cancer Res., 35, 1244.

ISRAEL, L. 1976. Immunochemotherapy with Corynebacterium parvum in disseminated cancer. Ann. NY Acad. Sci., 277, 241.

JACOBS, D., HOURI, M., LANDON, J. AND MERRETT, T.G. 1972. Circulating levels of immunoglobulin E in patients with cancer. Lancet, ii, 1059.

JACOBSON, J.B., GALUSKA, S. and STACKPOLE, C.W. 1978. In vivo modulations of thymus-leukaemia antigens on mouse leukaemia cells and thymocytes: retention of modulating antibody on the cell surface. J. Natl. Cancer Inst., 71, 819.

JAMES, K., MILNE, I., and WARR, G. 1974. Further studies on the effect of a transplanted methylcholanthrene-induced fibrosarcoma on humoral immunity in CBA mice. Clin. Exp. Immunol., 18, 243.

JAMES, K., CLUNIE, G.J.A., WOODRUFF, M.F.A, MCBRIDE, W.H., STIMSON, W.H. DREW, R. and CATTY D. 1975. The effect of Corynebacterium parvum therapy on immunoglobulin class and IgG subclass levels in cancer patients. Br. J. Cancer, 32, 310.

JAMES, K., WILLMOTT, N., MILNE, I. and MCBRIDE, W.H. 1976. Antitumor antibodies and immunoglobulin class and subclass levels in Corynebacterium parvum treated mice. J. Natl. Cancer Inst., 56, 1035.

JAMES, K., WILLMOTT, N., MILNE, I. and CULLEN, R. 1977a. Serological changes in adjuvant-treated mice; their specificity and relevance to tumor immunity. Cancer Immunol. Immunother., 2, 109.

JAMES, K., WOODRUFF, M.F.A., MCBRIDE, W.H. and WILLMOTT, N. 1977b. Serological changes associated with C.parvum treatment in nude mice. Br. J. Cancer, 35, 684.

JAMES, K., MCBRIDE, W.H. and STUART, A. Eds. 1977. The Macrophage and Cancer. Published by James, McBride and Stuart, Edinburgh.

JAMES, K., MILNE, I., MERRIMAN, J. and MCBRIDE, W.H. 1977. Further studies on anti-tumor responses induced by short-term pretreatment with syngeneic tumor cells. Br. J. Cancer, 39, 122.

JAMES ,K., MERRIMAN, J., MILNE, I., MCBRIDE, W.H. and IHLE, J.N. 1978. Tumor associated immunoglobulins, anti-tumor antibodies and anti-viral antibodies in C.parvum treated normal and tumor bearing mice. Cancer Immunol. Immunother., 25, 141.

JAMES, K., BESSOS, Y.H.I. and MERRIMAN, J. 1979. Association of host immuno-globulins with solid tumors in vivo. Br. J. Cancer, 40, 689.

JOHNSON, R.J., PASTERNACK, G.R. and SHIN, H.S. 1977a. Antibody mediated suppression of tumor growth. I. Suppression by murine IgG isolated from alloantiserum. J. Immunol., 118, 489.

JOHNSON, R.J., PASTERNACK, G.R. and SHIN, H.S. 1977b. Antibody mediated suppression of tumor growth. II. Macrophage and platelet cooperation with murine IgG1 isolated from alloantiserum. J. Immunol., 118, 494.

JOHNSON, R.J. PASTERNACK, G.R., DRYSDALE, B.E. and SHIN, H.S. 1977c. Antibody mediated suppression of tumor growth III. Molecular assay of murine IgG1 allo-antibody required to cause tumor suppression in vivo. J. Immunol., 118, 498.

JONDAL, M., SVEDMYR, E., and KLEIN, E. 1975. Killer T cells in a Burkitt's lymphoma biopsy. Nature, 255, 405.

JOSE, D.G. and SKVARIL, F. 1974. Serum inhibitors of cellular immunity in human neuroblastoma. IgG subclass of blocking activity. Int. J. Cancer, 13, 173.

JOSE, D.G. and SESHADRI, R. 1974. Circulating immune-complexes in human neuroblastoma: direct assay and role in blocking specific cellular immunity. Int. J. Cancer, 13, 824.

KALINSKE, R.W. and HOEPRICK, P.D. 1969. Engulfment and bactericidal capabilities of peripheral blood leukocytes in chronic leukaemias. Cancer, 23, 1094.

KAMO, I. and FRIEDMAN, H. 1977. Immunosuppression and the role of suppressive factors in cancer. Adv. Cancer. Res., 25, 271.

KEDAR, E., NAHAS, F., UNGER, E. and WEISS, D.W. 1978. In vitro induction of cell mediated immunity to murine leukaemia cells.III. Effect of methanol extraction residue fraction of BCG on the generation of cytotoxic lymphocytes against leukaemia. J. Natl, Cancer. Inst., 60, 1097.

KEISARI, Y. and WITZ, I.P. 1975. The specific blocking of humoral immune cytolosis mediated by anti-tumor antibodies degraded by lysosomal enzymes of tumor origin. Eur. J. Immunol, 5, 790.

KELLER, R., PEITCHEL, R., GOLDMAN, J.N. and GOLDMAN, M. 1976. An IgG Fc receptor induced in cytomegalo virus-infected human fibroblasts. J. Immunol., 116, 772.

KERBEL, R.S., PROSS, H.F. and ELLIOT, E.V. 1975. Origin and partial characterization of Fc-receptor bearing cells found within experimental carcinomas and sarcomas. Int. J. Cancer, 15, 918.

KIESSLING, R., KLEIN, E. and WIGZELL, H. 1975a. 'Natural' killer cells in the mouse.I. cytotoxic cells with specificity for mouse moloney leukaemia cells. Specificity and distribution according to genotype. Eur. J. Immunol., 5, 112.

KIESSLING, R., KLEIN, E., PROSS, H. and WIGZELL, H. 1975b. 'Natural' killer cells in the mouse.II. Cytotoxic cells with specificity for mouse moloney leukaemia cells. Characteristics of the killer cells. Eur. J. Immunol., 5, 117.

KIESSLING, R., PETRANYI, G., KARRE, K., JONDAL, M., TRACEY, D. and WIGZELL, H. 1976. Killer cells: a functional comparison between natural, immune T-cell, and antibody-dependent in vitro systems. J. Exp. Med. 143, 772.

KLEIN, E. 1972. Tumor immunology; escape mechanisms. Ann. Inst. Pasteur, 122, 593.

KLEIN, G. and KLEIN, E. 1977. Immune surveillance against virus-induced tumors and non rejectability of spontaneous tumors: contrasting consequences of host versus tumor evolution. Proc. Natl. Acad. Sci., 74, 2121.

KOPERSZTYCH, S., REZKALLAH, M.T., MIKI, S.S., NASPITZ, C.K. and MENDES, N.F. 1976. Cell-mediated immunity in patients with carcinoma. Cancer, 38, 1149.

KRONVALL, G., GREY, H.M. and WILLIAMS, R.C. 1970. Protein A reactivity with mouse immunoglobulins. J. Immunol., 105, 116.

LEWIS, M.G., HARTMAN, D. and JERRY, L.M. 1976. Antibodies and anti-antibodies in human malignancy: an expression of deranged immune regulation. Ann. NY Acad. Sci., 276, 316.

LIKHITE, V.V. 1974a. Rejection of tumors and metastases in Fischer 344 rats, following intratumor administration of killed Corynebacterium parvum. Int. J. Cancer, 14. 684.

LIKHITE, V.V. 1974b. The delayed and lasting rejection of mammary adenocarcinoma cell tumors in DBA/2 mice with use of killed Bordetella pertusis. Cancer Res. 34, 1027.

LOHMANN-MATHES, M.L., DOMZIG, W. and TASKOV, H. 1979. Antibody-dependent cellular cytotoxicity against tumor cells. I. Cultivated bone marrow derived macrophages kill tumor targets. Eur. J. Immunol. 9, 261.

MALUISH, A.E. and HALLIDAY W.J. 1975. Quantitation of anti-tumor cell-mediated immunity by a lymphokine-dependent reaction using small volumes of blood. Cell. Immunol., 17, 131.

MARTI, J.H. and THOMSON, D.M.P. 1976. Anti-tumor immunity in malignant melanoma assay by tube leucocyte adherence inhibition. Br. J. Cancer, 34, 116.

MARX, P.A. and WHEELOCK, E.F. 1976. Influence of immune stimulators on viral leukemogensis. Ann. NY Acad Sci., 276, 502.

MASTRANGELO, M.J., BEAD, D. and BELLET, R.E, 1976. Critical review of previously reported clinical trials of cancer immunotherapy with nonspecific immunostimulants. Ann. NY Acad. Sci., 277, 94.

McCOY, J.P., HOFHEINZ, D.E., NG, A.B.P., NORDQUIST, S., and HAINES, H.G. 1979. Tumor-bound immunoglobulin in human gynaecologic cancer. J. Natl. Cancer Inst., 63, 279.

McKNEALLY, M.F., MAVER, C., KANSEL. H.W. 1976. Regional immunotherapy of lung cancer with intrapleural BCG. Lancet, i, 377.

McRIPLEY, R.J., SELVARAJ, J., GLOVSKY, M.M. and SBARRA, A.J. 1967. The role of the phagocyte in host-parasite interactions. V. phagocytic and bactericidal activities of leukocytes from patients with different neoplastic disorders. Cancer Res., 27, 674.

MEDINA, D., SHEPHERD, F. and GROPP, T. 1978. Enhancement of tumorigenicity of preneoplastic mammary nodule lines by enzymatic dissociation. J. Natl. Cancer Inst., 60, 1121.

MEERS, P.D. 1973. Allergy and Cancer. Lancet, i, 884.

MILLER, G.A. and FELDMAN, J.D. 1977. Effect of macrophages and antibodies on in vivo growth of moloney sarcoma in the rat. J. Immunol., 119, 1445.

MOAV, N., HOCHBERG, Y., COHEN, G. and WITZ, I.P. 1978. Binding patterns of immunoglobulins from tumor-bearing mice to the corresponding tumor cells. J. Immunol. Methods, 22, 37.

MOLLER,G. and MOLLER, E. 1976. The concept of immunological surveillance against neoplasia. Transplant.Rev., 28, 3.

MOLLER, G. and MOLLER, E. 1978. Immunological surveillance against neoplasia. In ,Immunological Aspects of Cancer. Castro, J.E. ed. p. 205. MTP Press Ltd., Lancaster, England.

MOORE, M. and MOORE K. 1977. Kinetics of macrophage infiltration of experimental rat neoplasma. In ,The Macrophage and Cancer. James, K., McBride, B.and Stuart, A. eds. P. 330. Published by James, McBride and Stuart, Edinburgh.

MOORE, M. 1978. Antigens of experimentally induced neoplasms; a conspectus. In, Immunological Aspects of Cancer, Castro, J.E. ed., P.15. MTP Press, Ltd., Lancaster, England.

NISHIOKA, K., KAWAMURA, K., HIRAYAMA, J., KAWOSHIMA, T., SHIMATA, K.and KOGURE, M. 1976. The complement system in tumor immunity: significance of elevated levels of complement in tumor bearing hosts. Ann NY Acad Sci., 276, 303.

NORBURY, K.D. and KRIPKE, M. 1978. Ultraviolet Carcinogenesis in T cell depleted mice. J. Natl. Cancer Inst., 61., 917.

NORMAN, S.J. 1978. Tumor cell threshold required for suppression of macrophage inflammation. J. Natl. Cancer Inst., 60, 1091.

PENN, I. and STARZL, T.E. 1973. Immunosuppression and Cancer. Transp. Proc., 5, 943.

PICKERING, L.K., ANDERSON, B.C., CHOI, S. and FEIGIN, R.D. 1975. Leucocyte function in children with malignancies. Cancer, 35, 1365.

PIMM, M.V and BALDWIN, R.W. 1977. C.parvum immunotherapy of transplanted rat tumors. Int. J. Cancer., 20, 923.

PINSKY, C.M., HIRSHAUT, Y., WANEBO, H.J., FORTNER, J.G., MIKE, V., SCHOTTENFIELD, D., and OETTGEN, H.F. 1976. Randomized trial of Bacillus Calmette-Guerin (percutaneous administration) as surgical adjuvant immunotherpy for patients with stage II melanoma. Ann NY Acad. Sci., 277, 187.

PLATA, F., MACDONALD, H.R. AND ENGES, H.D. 1976. Characterization of effector lymphocytes associated with immunity to murine sarcoma virus (MSV) induced tumors. J. Immunol., 117, 52.

PRATHER, S.O., GELLER, R.W. and LANSCH, R.N. 1979. Inability of antiserum active in antibody-dependent cellular-cytotoxicity and arming tests to protect against simian virus 40 tumor cell challenge. J. Natl. Cancer Inst., 62, 1273.

PREHN, R.T. 1976. Do tumors grow because of the immune response of the host ? Transplant Rev., 28, 34.

PREHN, R.T. 1977. Immunostimulation of chemical oncogenesis in the mouse. Int. J. Cancer, 20, 918.

PRESTON, P.M. D'ARCY HART, P., and BROWN, I.N. 1977. Immunologically mediated macrophage aggregation in monolayers of peritoneal cells from BCG-sensitized mice. Immunology, 32, 33.

PRICE, M.R. and BALDWIN, R.W. 1977. Tumor-specific complement-dependent serum cytotoxicity against a chemically induced rat hepatoma. Int. J. Cancer, 20, 284.

PRICE, M.R. and ROBINS, R.A. 1978. Circulating factors modifying cell-mediated immunity in experimental neoplasia. In, Immunological Aspects of Cancer. Castro, J.E., ed. p. 155. MTP Press Ltd., Lancaster, England.

PRICE, M.K., HOFFKEN, K. and BALDWIN, R.W. 1979. Activity of syngeneic complement for revealing antibody-induced cytotoxicity against a rat hepatoma. Transplantation, 28, 140.

PRICE, M.R., DENNICK, R.G., ROBINS, R.A. and BALDWIN, R.W. 1979. Modification of the immunogenicity and antigenicity of rat hepatoma cells I. cell-surface stabilization with glutaraldehyde. Br. J. Cancer, 39, 621.

PROSS. H.F. and KERBEL, R.S. 1976. An assessment of intratumor phagocytic and surface marker bearing cells in a series of authochthonous and early passaged chemically induced murine sarcomas. J. Natl. Cancer Inst., 57, 1157.

RAN, M., KLEIN, G. and WITZ, I.P. 1976. Tumor bound immunoglobulins. Evidence for the in vivo coating of tumor cells by potentially cytotoxic anti-tumor antibodies. Int. J. Cancer, 17, 90.

RAN, M. YAKUBOWICZ, M. and WITZ, I.P. 1978. Lymphocytotoxic antibodies eluted from in vivo propagating sarcoma cells of mice: Brief Communication. J. Natl. Cancer Inst., 60, 1509.

RHODES, J. 1977. Regulatory effects of normal human monocytes and monocytes activated in cancer on normal lymphocyte responses to mitogen. In, The Macrophage and Cancer. James, K., McBride, B.and Stuart A. eds. p. 390. Published by James, McBride and Stuart, Edinburgh.

RITTENHOUSE, H.G., AR, D., LYNN, M.D. and DENHOLM, D.K. 1978. The spontaneous release of a high-molecular weight aggregate containing immunoglobulin G from the surface of Ehrlich ascites tumor cells. J. of Supramolecular Structure, 9, 407.

RIOS, A. and SIMMON,S R.L. 1976. Experimental cancer immunotherapy: modification of tumor cells to increase immunogenicity. Ann NY Acad. Sci., 276, 45.

ROBERTS, M.M, BASS, E.M., WALLACE, I.W.J. and STEVENSON, A. 1973. Local immunoglobulin production in breast cancer. Br. J. Cancer, 27, 269.

ROBINS, R.A. 1975. Serum antibody responses to an ascitic variant of rat hepatoma D23. Br. J. Cancer, 32, 21.

ROSENBAUM, J.T. and DWYER, J.M. 1977. The role of IgE in the immune response to neoplasia: a review. Cancer, 39, 11.

RUSSELL, S.W., DOC, W.F., HOSKINS, R.G. and COCHRANE, C.G. 1976a. Inflammatory cells in solid murine neoplasma. I. tumor disaggregation and identification of constituent inflammatory cells. Int J. Cancer, 18, 322.

RUSSELL, S.W., GILLESPIE, G.Y., HANSEN, C.B. and COCHRANE, C.G. 1976b Inflammatory cells in solid murine neoplasms II. Cell types found throughout the course of moloney sarcoma regression or progression. Int J. Cancer, 18, 331.

RUTHERFORD, J.C., WALTERS, B.A.J., CAVAYE, G. and HALLIDAY, W.J. 1977. A modified leucocyte adherence inhibition test in the laboratory investigation of gastrointestinal cancer. Int J. Cancer, 19, 43.

RYGAARD, J. and POVLSEN, C.O. 1976. The nude mouse vs the hypothesis of immunological surveillance. Transplant. Rev., 28, 43.

SADLER, T.E. and CASTRO, J.E. 1978. Experimental nonspecific immunotherapy. In, Immunological aspects of cancer. Castro, J.E. ed. p. 357 MTP Press Ltd., Lancaster, England.

SEGERLING, M., OHANIAN, S.H. and BORSOS, T. 1976. Persistence of immunoglobulin and complement components C_4 and C_3 bound to guinea pig tumor cells. J. Natl. Cancer Inst., 57, 145.

SHAPIRO, S., HEINONEN, O.P. and SISKIND, V. 1971. Cancer and Allergy. Cancer, 28, 396.

SHEARER, W.T. and FINK, M.P. 1977. Immune surveillance system: its failure and activation. In, Progress in Haematology, Vol. X; Brown, E.B., ed. Grune and Stratton, U.S.A.

SHIMKIN, M.B. 1977. Contrary to Nature. p. 381. DHEW publication, Washington, U.S.A.

SHIN, H.S., JOHNSON, R.J., PASTERNACK, G.R. and ECONOM, J.S. 1978 Mechanisms of tumor immunity: the role of antibody and non-immune effectors. In, Progress in Allergy. Kallos, P., Waksman, B.H. and DeWeck, A.L. eds. p. 163, 25 S.Karger, A.G., Basel, Switzerland.

SHUSTER, J., THOMSON, D.M.P. and GOLD, P. 1978. Immunodiagnosis. In, Immunological Aspects of Cancer, Castro, J.E. ed. p. 283. MTP Press Ltd., Lancaster, England.

SKEEL, R.T., YANKEE, R.A. and HENDERSON, E.S. 1971. Hexose monophosphate shunt activity of circulating phagocytes in acuate lymphocytic leukaemia. J. Lab. Clin Med., 77 ,975.

SMALL, M., TRAININ, N. 1976. Separation of populations of sensitized lymphoid cells into fractions inhibiting and fractions enhancing syngeneic tumor growth in vivo. J. Immunol., 117, 292.

SOYKA, L.F., HUNT, W.G., KNIGHT, S.E. and FOSTER, R.S. 1976. Decreased liver and lung drug-metabolizing activity in mice treated with C.parvum. Cancer Res., 36, 4425.

SPARKS, F.C., TING, C.C. , HAMMOND, W.G. and HERBERMAN, R.B. 1969. An isotopic antiglobulin technique for measuring antibodies to cell-surface antigens. J. Immunol., 102, 842.

SPARKS, F.C. O'CONNELL, T.X., LEE, Y.T.N. and BREEDING, J.H. 1974. BCG therapy given as an adjuvant to surgery: prevention of death from metastases from mammary adenocarcinoma in rats. J.Natl. Cancer Inst., 53, 1825

STEELE, G. Jr., AHKERST, H.O., SJOGREN, J.V. and LANNERSTAD, O. 1975. Absorption of blocking activity from human tumor-bearer sera by Staphylococcus aureus, cowan I. Int J. Cancer, 15, 180.

STRAUSS, R.R., PAUL, B.B., JACOBS, A.A., SIMMONS, C. and SBARRA, A.J. 1970. The metabolic and phagocytic activities of leucocytes from children with acute leukaemia. Cancer Res., 30, 480.

STUTMAN, O. 1975. Immunodepression and malignancy. Adv. Cancer Res., 22, 261.

SZYMANIEC, S., and JAMES, K. 1976. Studies on the Fc-receptor bearing cells in a transplanted methylcholanthrene induced mouse fibrosarcoma. Br. J. Cancer, 33, 36.

TAKASUGI, M., MICKEY, M.R. and TERASAKI, P.I. 1973. Reactivity of lymphocytes from normal persons on cultured tumor cells. Cancer Res., 33, 2898.

TAMERIUS, J., HELLSTROM, I., and HELLSTROM, K.E. 1975. Evidence that blocking factors in the sera of multiparous mice are associated with immunoglobulins. Int J. Cancer, 16, 456.

TAN, C.V., ROSNER, F. and FELDMAN, F. 1973. Nitroblue tetrazolium dye reduction. N.Y. State J. Med., 73, 952.

TERMAN, D.S., STEWART, I., TAVEL, A. and KIRCH, D. 1975. Localization of neuroblastoma in vivo with tumor specific antibodies. Cancer Res., 35, 1761.

TING, C.C. 1976. Detection of anti-tumor antibodiy in virally induced tumors and its relationship to tumor growth. Int. J. Cancer, 18, 205.

TONDER, O., ENGIKOLI, C.K., JEWELI, W.R., MORSE, P.A. and HUMPHREY L.J. 1976. Tumor Fc-receptors and tumor-associated immunoglobulins. Acta. Path. Microbiol. Scand. Sect. A. 84, 105.

TORISU, M., FUKAWA, M., MISHIMURA, M., HARASAKI, H., KAI, S. and TANAKA, J. 1976. Immunotherapy of cancer patients with Bacillus Calmette Guerin: summary of four years of experience in Japan. Ann. NY Acad. Sci., 277, 160.

URE, D.M.J. 1969. Negative association between allergy and cancer. Scot. Med. J., 14, 51.

VAN EPPS, D.E., REED, K., and WILLIAMS., R.C. 1978. Suppression of human PMN bactericidal activity by human IgA paraproteins. Cell Immunol.,36, 363.

VAN FURTH, R. 1977. Concluding remarks. In, The Macrophage and Cancer. James, K., McBride, B., and Stuart, A. eds. p.445. Published by James, McBride and Stuart, Edinburgh.

VANKY, F., TREMPE, G., KLEIN, E., and STJERNSWARD, J. 1975. Human tumor-lymphocyte interaction in vitro: blastogenesis correlated to detectable immunoglobulin in the biopsy. Int. J. Cancer., 16 , 113.

VAN SNICK, J.L. and MASSON, P.L. 1980. Incidence and specificities of IgA and IgM anti-IgG auto-antibodies in various mouse strains and colonies. J. Exp. Med., 151, 45.

VIDEBACK, A. 1973. Leukaemia and Cancer in relation to immunosuppressive therapy. Scan. J. Haemat., 10, 241.

WEISS, D.W., ZUPERMAN, O., FATHALLAH, N. and KEDAR, E. 1976. Mode of action of mycobacterial fractions in anti-tumor immunity: preliminary evidence for a direct nonspecific stimulatory effect of MER on immunologically reactive cells. Ann. NY Acad. Sci., 276, 536.

WEISS, D.W. 1977. The questionable immunogenecity of certain neoplasma. Cancer. Immunol. Immunother., 2, 11.

WIEPJES, G.J. and PROP, F.J.A. 1970. Improved method for preparation of single cell suspensions from mammary glands of adult virgin mice. Exptl, Cell. Res., 61. 451.

WESTMORELAND, D. and WATKINS, J.F. 1974. The IgG receptor induced by Herpes simplex virus: studies using radioiodinated IgG. J. Gen. Virol.,24, 167.

WITZ, I.P., KINAMON, S., RAN, M. and KLEIN, G. 1974. Tumor-bound immunoglobulins. The in vitro fixation of radioiodine-labelled anti-immunoglobulin reagents by tumor cells. Clin. Exp. Immunol, 16, 321.

WITZ, I.P., LEE, N. and KLEIN, G. 1976. Serologically detectable specific and cross-reactive antigens on the membrane of a polyoma virus induced murine tumor. Int. J. Cancer., 18, 243.

WITZ, I.P. 1977. Tumor-bound immunoglobulins: in situ expressions of humoral immunity. Adv. Cancer Res., 25, 95.

WITZ, I.P. and MOAV, N. 1978. Characterization of immunoglobulins eluted from murine tumor cells: binding patterns of cytotoxic anti-tumor IgG. J. Immunol. Methods., 22, 51.

WOLOSIN, L.B. and GREENBERG, A.H. 1979. Murine natural anti-tumor antibodies. I. rapid in vivo binding of natural antibody by tumor cells in syngeneic mice. Int J. Cancer, 23, 519.

WOODRUFF, M.F.A. and BOAK, J.L. 1966. Inhibitory effect of injection of Corynebacterium parvum on the growth of tumor transplants in isogenic hosts. Br. J. Cancer, 20, 345.

YEENOF, E., WITZ. I.P. and KLEIN, E. 1976. Interaction of antibody and cell surface localized antigen. Int. J. Cancer, 17, 633.

ZINZAR, S.N., SVET-MOLDAVSKY. G.J.and KARMAROVA, N.V. 1976. Non-immune and immune surveillance. I. growth of tumors and normal foetal tissues grafted into newborn mice. J. Natl. Cancer Inst., 57, 47.

ZINZAR, S.N., SVET-MOLDAVSKY, G.J. and KARMAROVA, N.V. 1978. Non-immune and immune surveillance II. Effect of recipient's age tumor immunogenecity and neonatal thymectomy on tumor growth inhibition. J. Natl. Cancer Inst., 61, 737.